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13. ABSTRACT ***(Maximum 200 Words)***

In the third and final year of this three year project, we have characterized 2-crotonyl-oxymethyl-2-cyclohexenone (COMC-6) and its derivatives as substrates for human glutathione transferase (hGST), producing as products cytotoxic alkylating agents of nucleic acids and/or proteins critical to cell function. Compounds of this type should be potent antitumor agents against breast cancer cells over expressing GST as part of the multidrug resistance phenotype. Specifically, we have (a) discovered that hGSTP1-1 efficiently catalyzes the conversion of COMC-6 to a reactive exocyclic enone of COMC-6, (b) shown that the exocyclic enone can alkylate oligonucleotides *in vitro* –the probable basis of antitumor activity (c) synthesized and characterized the 5- and 7-membered ring homologues of COMC-6 (COMC-5, COMC-7) and demonstrated that COMC-6 is more potent to B16 tumor cells than COMC-6 *in vitro*, and (d) demonstrated that COMC-6 displays similar toxicities to human colon HT29 versus HT29 over-expressing the phosphoglycoprotein responsible for some type of multidrug resistance. We are now in the process of testing the *in vitro* tumoricidal activities of COMC-6 and its homologes to MCF-7 breast tumor cells versus MCF-7 over-expressing hGSTP1-1. *Key words: glyoxalase I, glutathione transferase, N-hydroxycarbamoyl esters of glutathione, 2-.*

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Introduction

The objective of the proposed research program is to develop a novel new class of anti-tumor agents that will, in effect, turn the enzymes that normally confer resistance to anticancer drugs into "drug factories" inside breast cancer cells. The proposed anti-tumor agents do not specifically target rapidly dividing cells and, therefore, might not exhibit the side effects so commonly associated with cancer chemotherapy. The proposed agents are cyclic N-hydroxycarbamates that will capitalize on differences in the activities of glutathione (GSH)-dependent enzymes between normal cells and cancer cells in order to generate high levels of selective tumoricidal activity. Specifically, the carbamates are designed to function as cell-permeable substrates for the p-isoform of glutathione transferase (hGSTP1-1). The resulting N-hydroxycarbamoyl esters of GSH are predicted to serve both as powerful mechanism-based competitive inhibitors of the detoxifying enzyme glyoxalase I (GlxI), and as slow substrates for the thioester hydrolase glyoxalase II (GlxII). The hypothesis that the πGST substrates will selectively inhibit the growth of breast tumor cells versus normal cells is founded on three observations. First, GlxI plays a critical detoxification role in cells by converting the metabolite methylglyoxal to nontoxic *D*-lactate. Therefore, inhibitors of GlxI should inhibit cells by causing the build-up of intracellular methylglyoxal. Second, GST is often expressed at *higher* levels in human breast tumors than in peritumoral tissues. This is particularly true of cells exhibiting the multi-drug resistance phenotype. Thus, the GlxI inhibitors should be generated more rapidly in tumor cells exposed to the GST substrates.

During the course of these studies, we discovered that the known antitumor agent 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) also belongs to this class of compounds in a very unsuspected way. This compound and its homologues are the focus of the third year progress report given below. The original aims of this project were as follows: (1.) To synthesize and test cyclic compounds of the following general type as substrates for πGST: YCON(OH)R, where Y equals different leaving groups and R equals different alkyl or aryl substituents. (2.) To determine the competitive inhibition constants of the resultant GSH-conjugates (GSCON(OH)R) with GlxI and the kinetic properties of the conjugates as substrates for GlxII. (3.) To determine the rates at which YCON(OH)R diffuse into MCF7 human breast cancer cells and Adr^R MCF7 cells (over-expressing hGSTP1-1), and form GlxI inhibitors of the type GSCON(OH)R. (4.) To measure the efflux rates of GSCON(OH)R from MCF7 and Adr^R MCF7 cells. (5.) To evaluate the IC_{50}s of YCON(OH)R with MCF7 and Adr^R MCF7 cells for comparison with the intracellular concentrations of GSCON(OH)R with these cell lines.

Body

I *Overview:* The "*overview*" section of this final progress report briefly describing the work accomplished over the course of this three year project. The "*detailed account*" section is designed for those interested in the technical aspects of our work.

During years 1 and 2 of this project, we have accomplished tasks 1 and 2 related to the synthesis and testing of compounds that serve as substrates for glutathione transferase, producing as products tight-binding inhibitors of the methylglyoxal detoxifying enzyme glyoxalase I:

Task 1. (completed):To identify substrates for glutathione transferase (GST) that give as products inhibitors of glyoxalase I.

Task 2. (completed):To determine the competitive inhibition constants of the resulting products of the GST reaction as inhibitors of glyoxalase I and as substrates for glyoxalase II.

Published papers describing this work can be found in Appendix I (papers 1 and 2). This work provides the experimental and conceptual foundation for a fresh approach to inhibiting breast tumors that exhibit the multi-drug resistance phenotype.

At the end of the first year's work we also asked and received approval to pursue an additional task that arose from a collaboration between this laboratory and Professor Bruce Ganem's laboratory at Cornell University. This task is related to accomplished tasks 1 and 2 and expands the number of compound we are testing for tumoricidal activity against breast cancer:

Task 5 (completed): To evaluate the antitumor agent 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) as a substrate for GST, producing as a product a glutathione adduct that is an inhibitor of glyoxalase I.

Papers describing this work may be found in Appendix I (papers 3-8). In the PI's judgment, this is some of the most important work to come out of the second and third year of this research program, because these studies *overturn* the accepted view that the antitumor activity of COMC-6 is exclusively due to inhibition of glyoxalase I. Rather, the antitumor activity of COMC-6, and compounds like it, most likely arise primarily from the formation of a highly reactive exocyclic enone that is formed during the GST-catalyzed conversion of COMC-6 to its corresponding glutathione adduct. We believe that cytotoxicity arises from the covalent modification of cellular nucleic acids and/or proteins by the exocyclic enone. So What? This implies that breast tumor cells that over-express GST, as part of the multi drug resistance phenotype, will be particularly sensitive to COMC-6 in comparison to peritumoral tissue that expresses low levels of GST. In addition to the compounds described in the original proposal, we will also test the selective tumoricidal activity of COMC-6 *in vitro* in collaboration with Dr. Eiseman.

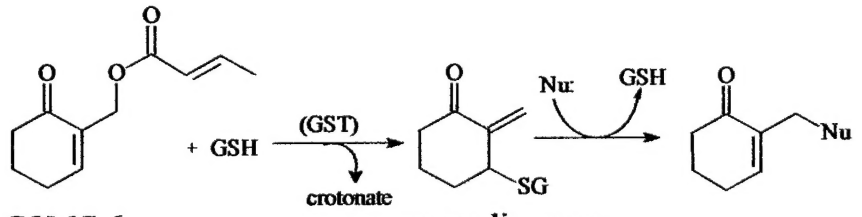
In the third year, we have further evaluated the molecular basis of the ability of GST to catalyze the conversion of COMC-6 to a reactive exocyclic enone, on the basis of molecular docking studies between the GSH adduct of COMC-6 and human GST (hGSTP1-1). Moreover, we have established that the exocyclic enone can alkylate model oligonucleotides, the likely basis of the antitumor activity of COMC-6. We have also published a preliminary study of the membrane transport properties and tumoricidal activities of COMC-6 and two homolgues of COMC. This work is related to accomplishing tasks 3 and 4, given in the original research proposal:

Task 3. (completed): To measure the influx and efflux rates of the GST substrates with breast cancer cells.

Task 4. (partially completed): To evaluate the IC_{50} values of the GST substrates with breast cancer cells.

We continue to work on task 4 aimed at evaluating the differential toxicities of COMC-6 derivatives toward MCF-7 breast tumors that over express or under express hGSTP1-1. We have applied for and received a one-year extension of this project, to complete these studies. This should take about 3 months. We will then file a supplement to our final report describing the results of these studies. This work is being done in collaboration with Dr. Julie Eiseman at the University of Pittsburgh Cancer Center, as originally planned.

II. *Detailed account*. In the third year of this three year project we have further evaluated the hypothesis that the known tumoricidal activities of COMC-6 is due, in part, to its glutathione transferase (GST)-catalyzed conversion to a highly reactive exocyclic enone, which kill cells by reacting with intracellular proteins and/or nucleic acids, **Eqn. 1**.



COMC-6 exocyclic enone (1.

where GSH = glutathione (γ-Glu-Cys-Gly); Nu: = nucleophiles (DNA, proteins)

If correct, compounds of this type might show exceptionally high levels of tumoricidal activity against tumor cells overexpressing GST, as part of the multi-drug resisitance phenotype, Table 1.

The above hypothesis emerged from our recent discovery that COMC-6 is an excellent substrate for hGSTP1-1 and forms covalent adducts with model dinucleotides according to Eqn 1.

Table 1. Altered GST Expression in Drug-Resistant and Drug-Sensitive Cell Lines

Drug	Fold Resistance	Cell line	Cross-resistance	Changes in GST
Adriamycin	100	MCF-7 human breast (Adr^r)	Actinomycin D, vinblastine	45-fold increase in hGSTP1
Adriamycin	32	H69AR human small cell lung	Colchicine, daunomycin	10-fold increase in hGSTP1
Adriamycin	75	SW620-ADR human colon	Actinomycin D, puromycin	2-fold increase in hGSTP1
Adriamycin	13	Friend erythroleukemia (ARN2)	Mitoxanthrone, vincristine, VP-16	>10-fold increase in mGSTA1 or A2
Adriamycin	84	P388 murine leukemia	ns	2.2-fold increase in mGSTP1
Adriamycin	200	MatB13762 rat mammary (Adr^r)	Vincristine	5-fold increase in rGSTA3 and P1
Arsenic	9	SA7 Chinese hamster ovary	ns	Levels of GSTP1 correlate with resistance
BCNU	3.5	9L rat gliosarcoma (9L-2)	CNDP	>2-fold increase in rGSTM1 or M2
Bleomycin	0.03	Chinese hamster ovary (BL-10)	ns	Sensitivity normalized by hGSTA1
CDNB	3	H322 human lung ($CDNB^r$)	CuOOH	>10-fold increase in hGSTA1 and A2
Chlorambucil	15	Walker 256 rat mammary	phosphoramide mustard	>10-fold increase in rGSTA3
Chlorambucil	24	Chinese hamster ovary ($CHO\text{-}Chl^r$)	Mechlorethamine, melphalan	>10-fold increase in hamster GST Yc
Chlorambucil	>10	Mouse fibroblast 3T3 (N50-4)	ns	>10-fold increase in alpha class GST
Cisplatin	18	HeLa human cervix (HeLa-CPR)	ns	6-fold increase in hGSTA1 and A2
Cyclophospham	19	Yoshida rat sarcoma (YR cyclo)	Phosphoramide mustard	6-fold increase in CDNB activity
EA	2.5	MCF-7 human breast (EA^r)	ns	> 10-fold increase in mu-class GST
EA	2	HT 29 human colon (HT/M)	ns	3-fold increase in hGSTP1 mRNA
Etoposide (VP-1	14	MCF-7 human breast (VP6E)	Vincristine	>10-fold increase in hGSTP1
Hepsulfam	10	MCF-7 human breast	Adriamycin	>10-fold increase in hGSTP1
Hepsulfam	8	Hs578T human breast	ns	Levels of hGSTP1 correlate with resistance
Melphalan	17	HS-Sultan human plasma cell	ns	1.5-fold increase in hGSTP1
Melphalan	10	MatB13762 rat mammary(Mln^r)	BCNU, ionizing radiation	10-fold increase in rGSTA3 and P1
Mitomycin C	4.5	SCaBER human bladder	ns	5-fold increase in hGSTP1
Mitoxantrone	200	Caco-2 human colon	ns	>6-fold increases in hGSTP1, A1, and A2
MNNG	ns	Rat liver endothelial cells (GP9TA)	Adriamycin	10-fold increase in rGSTP1
Novantrone	5	MCF-7 human breast (NOV6E)	ns	>10-fold increase in hGSTP1
Oxazaphosphori	6	MCF-7 human breast (MCF/HC)	ns	2.7-fold increase in GST
TGF-β1	ns	WB-F344 rat liver epithelial cells	Adriamycin, melphalan	1.5-fold increase in CDNB activity
Vincristine	3	MCF-7 human breast (VCR6E)	VP-16	>10-fold increase in hGSTP1
Vincristine	11	MCF-7 human breast (VCREMS)	Adriamycin, VP-16	>10-fold increase in hGSTP1

Taken from: Hayes, D. and Pulford, D. J. (1995), Crit Rev. in Bioch. Mol. Biol. *30*, 445-600. Most cell lines were derived by *in vitro* selection. BCNU, 1,3-Bis 2-chloroethyl-1-nitrosourea; CNDP, 2-[3-(Chloroethyl)-3-nitrosoureido]-*D*-dioxyglucopyranose; ns, not stated, VP-16, etoposide; CuOOH, cumene hydroperoxide; EA, ethacrynic acid.

A. Inhibition of glyoxalase I by the glutathione (GSH) adduct of COMC-6 is unlikely to account for the cytotoxicity of COMC-6 [Joseph et al.. (2000 a); Joseph et al.. (2000 b) Appendix I]. Previous workers have long thought that the antitumor activity of COMC-6 was due to inhibition of glyoxalase I by the GSH adduct of COMC-6, resulting in the build up of cytotoxic methylglyoxal in tumor cells. Indeed, we have demonstrated that transition state analog inhibitors of glyoxalase I retard the growth of both murine and human tumors in culture.[1] The K_i values with human glyoxalase I are in the submicromolar concentration range. In addition, the IC_{50} values are approximately proportional to the K_i values, such that a relatively weak enzyme inhibitor with a K_i = 0.16 μM gives an IC_{50} value >100 μM. On this basis, COMC-6 should exhibit very poor potency, because the corresponding GSH conjugate is a weak inhibitor of the enzyme (K_i = 106 μM). This is contrary to reported IC_{50} values in the range 0.5-19 μM for established murine and human tumor cell lines.[2] We have confirmed in our laboratories that COMC-6 is indeed a very potent inhibitor of B16 melanotic melanoma in culture (IC_{50} = 0.04 μM), Fig. 1.

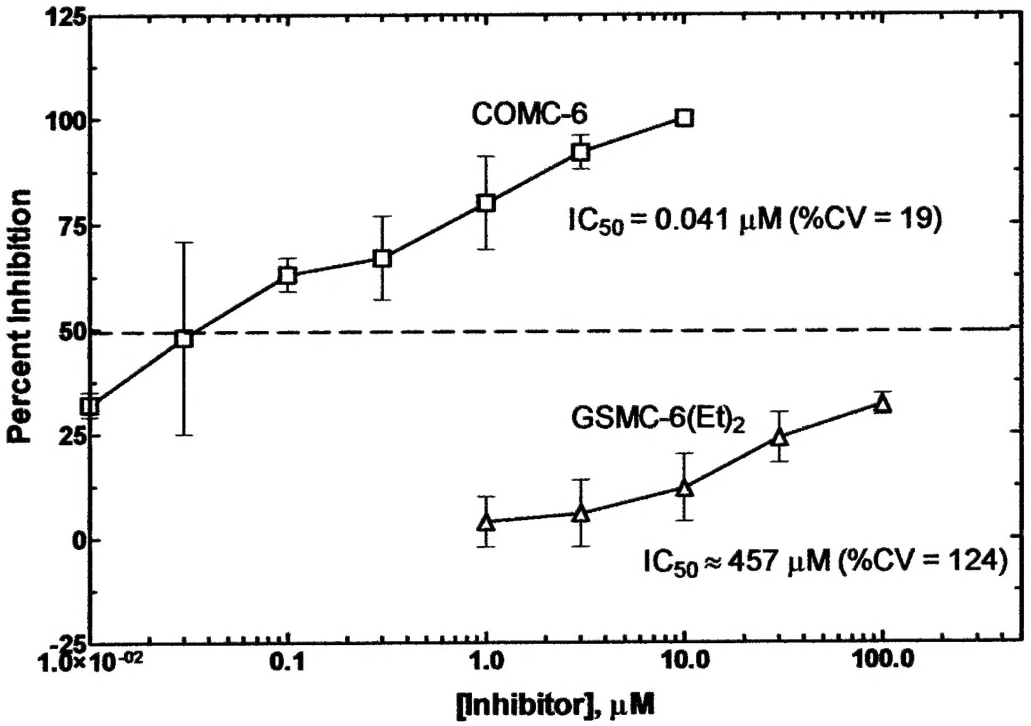


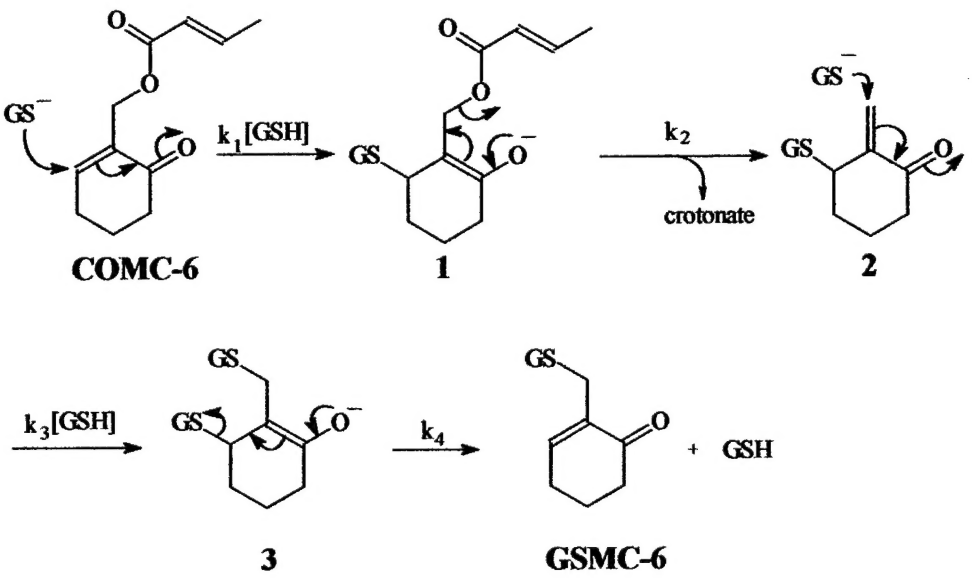
Fig. 1. *Growth inhibition of B16 cells in the presence of COMC-6 and GSMC-6(Et)$_2$. Methods: B16 (2 x 10^4 cells) were plated in 24 well plates containing RPMI 1640/10% bovine calf serum, 10 μg/ml gentamicin and incubated at 37°C under an atmosphere of 5% CO_2 and 95% humidified air. Drug was added at the indicated concentrations. After 72 h, cells were trypsinized, concentrated and counted by trypan blue exclusion using a hemocytometer. IC_{50} values are the mean ± standard deviation of triplicate determinations carried out in 3 separate assays on different days. IC_{50} values were calculated using the Hill equation and the program Adapt.*

Moreover, when the GSH conjugate of COMC-6 is indirectly delivered into B16 cells as the [glycyl, glutamyl] diethyl ester GSMC-6 (Et)$_2$ (a prodrug strategy developed in this laboratory)[1] there is little inhibition of cell growth (IC_{50}> 400 μM), Fig.1. *This suggests that cytotoxicity does not arise simply from the presence of the conjugate inside the cells, but must be due either to unconjugated COMC-6 or to a reactive intermediate formed during conjugate addition of GSH to COMC-6.*

B. The Reaction of COMC-6 with GSH Involves the Formation of a Reactive Intermediate, on the Basis of Kinetic Measurements and Chemical Trapping Experiments [Hamilton et al. (2002), Appendix I]. A variety of mechanisms have been proposed to account for the reaction of COMC with GSH. On paper, the simplest mechanism is a direct, S_N2 displacement of the crotonate ester by the nucleophilic thiol of GSH. However, carboxylic esters are only moderate leaving groups, and the α,β-unsaturated enone system would be expected to undergo much more rapid conjugate addition reactions with thiols. Thus, a stepwise 1,4-addition/β-elimination mechanism can be envisioned in which GSH first adds to the endocyclic enone function of COMC-6 via

enolate **1** to form an intermediate exocyclic enone **2.** The exocyclic enone then subsequently reacts with another molecule of GSH to give the endocyclic enone **3** (Scheme 1). Exocyclic enones like **2** are particularly reactive Michael acceptors, and could function as carcinostatic agents by reacting with nucleic acids and proteins critical to cell function.

Scheme 1



Kinetic studies. Experimentally, the reaction of excess GSH with **1a** to give **1e** follows a simple first-order decay with no evidence of any intermediate (Fig. 2, trace A):

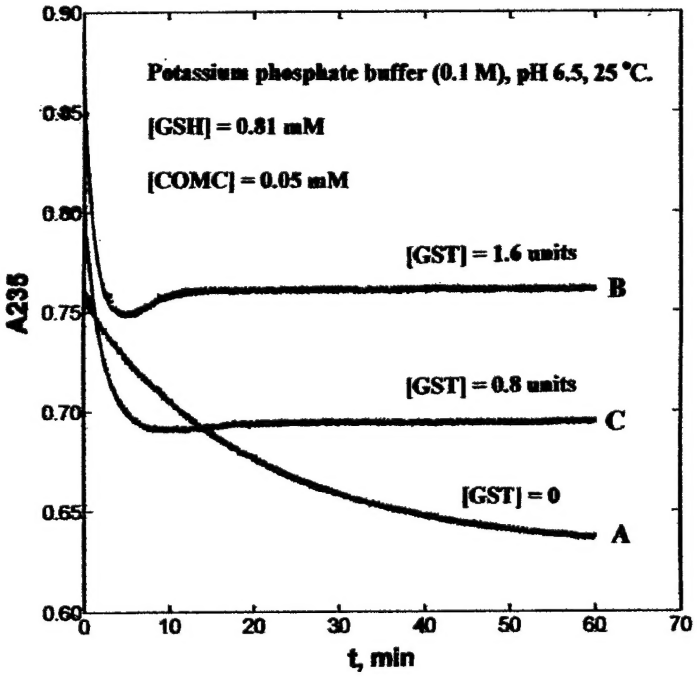


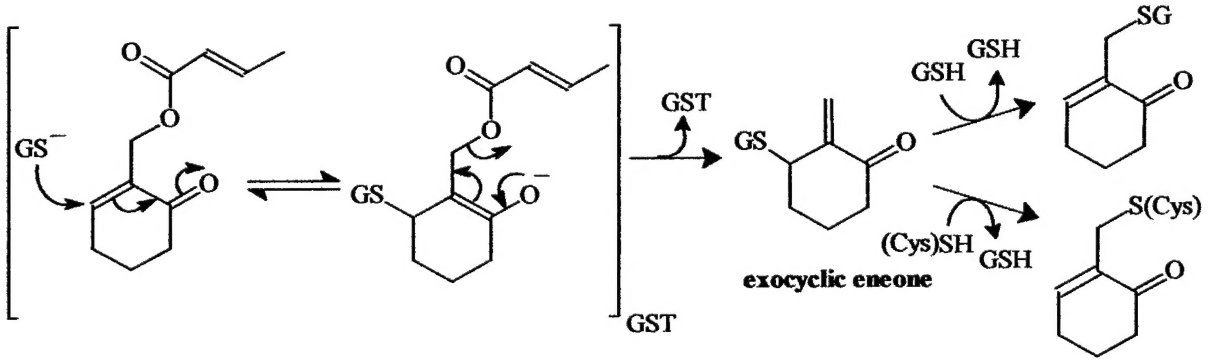
Fig. 2. *Time course for the loss of COMC-6 (0.05 mM) in the presence of GSH (0.81 mM) (A) in the absence of GST (k = 0.0514 ± 0.0002 min^{-1}) and (B) in the presence of 1.6 units of GST (k_1 = 0.641 ±0.019 min^{-1}; k_2 = 0.315 ± 0.015 min^{-1}) and (C) 0.8 units of GST (k_1 = 0.451 ± 0.0063 min^{-1}; k_2 = 0.135 ±0.010 min^{-1}).* Different end-point absorbances are due to different background absorbances of enzyme protein. Conditions: phosphate buffer (0.05 M), pH 6.5, 25 °C

Nevertheless, this observation would still be consistent with the mechanism shown in Scheme 1, provided that the formation of the exocyclic enone is rate determining.

During the course of these studies, we made the chance observation that in the presence of human placental glutathione transferase (pi-GST) the rate of conversion of COMC-6 to GSMC-6 is composed of a rapid, enzyme-dependent, initial phase and a slower enzyme-independent first-order phase (Fig. 4, traces B and C). This can be explained in terms of the mechanism shown in Scheme 1, wherein pi-GST catalyzes the rapid formation of exocyclic enone **2**, which subsequently reacts with free GSH in solution to give GSMC-6. The shape of the kinetic trace indicates that the molar absorptivity of **2** is less than that of GSMC-6.

Intermediate trapping. In order to confirm that the exocyclic enone is the immediate product of catalysis, the transferase reaction was carried out in the presence of equimolar amounts of GSH and cysteine, and the ratio of the two thiol addition products determined by reverse-phase HPLC, Scheme 2.

Scheme 2



Judging from the ratio of first-order rate constants for the enzymic and nonenzymic processes, the amount of enzyme-derived product formed in the enzyme-catalyzed pathway was estimated to be about 90 percent of the total product yield. Moreover, the interconversion of thiol adducts proved minimal: in the presence of cysteine (2.5 mM), glutathione adduct GSMC-6 (0.5 mM) formed less than 1% of the cysteine adduct over 27 h, as determined by HPLC. Thus, the product ratio observed in the enzymic reaction must reflect the true relative rates of reaction of **2** with GSH and cysteine. If the exocyclic enone **2** dissociates from the surface of the enzyme and reacts with the free thiols in solution, the product ratios for the enzymic and nonenzymic processes should be identical, as was observed to be the case.

Isolation of the exocyclic enone 2. The chemical identity of the intermediate was confirmed by briefly incubating COMC-6 in the presence of GSH and GST, quenching the reaction mixture in dilute acetic acid, and resolving the components by reverse-phase HPLC, as shown in Fig. 3.

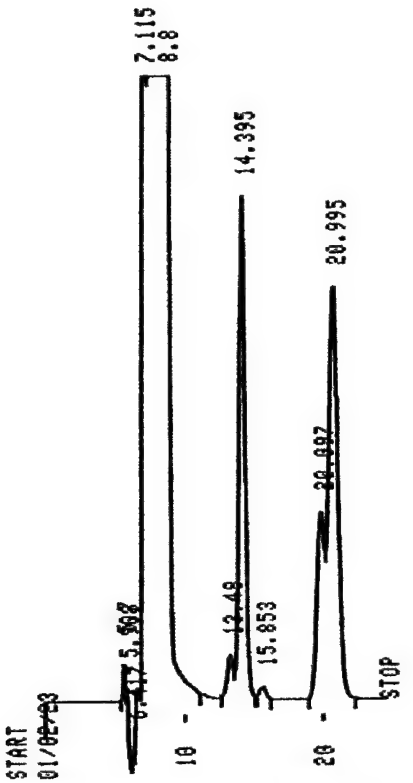


Fig. 3. *Elution profile from a reverse-phase HPLC column of a reaction mixture 40 seconds after combining GSH (0.5 mM), GST, and COMC-6 (0.1 mM) in 0.1 M phosphate buffer pH 6.5.* The peak at 21 min corresponds to the the GSH adduct GSMC-6, the peak at 20.1 min is tentatively identified as the exocyclic enone **2,** and the peak at 14.4 min. is crotonic acid. Running solvent: 5% methanol in water containing 0.5% acetic acid.

The compound corresponding to the 20.1 min peak is identified to be the exocyclic enone **2**. When neutralized and combined with cysteine, this compound gives a new peak in the chromatogram that comigrates with authentic cysteine adduct. Moreover, the first order rate constant leading to GSMC-6 is similar in magnitude to that associated with the slow phase of the transferase-catalyzed conversion of COMC-6 to GSMC-6, Fig. 2. The 600 MHz ^{1}H NMR spectrum of the putative intermediate species is consistent with exocyclic enone **2**, Fig. 4.

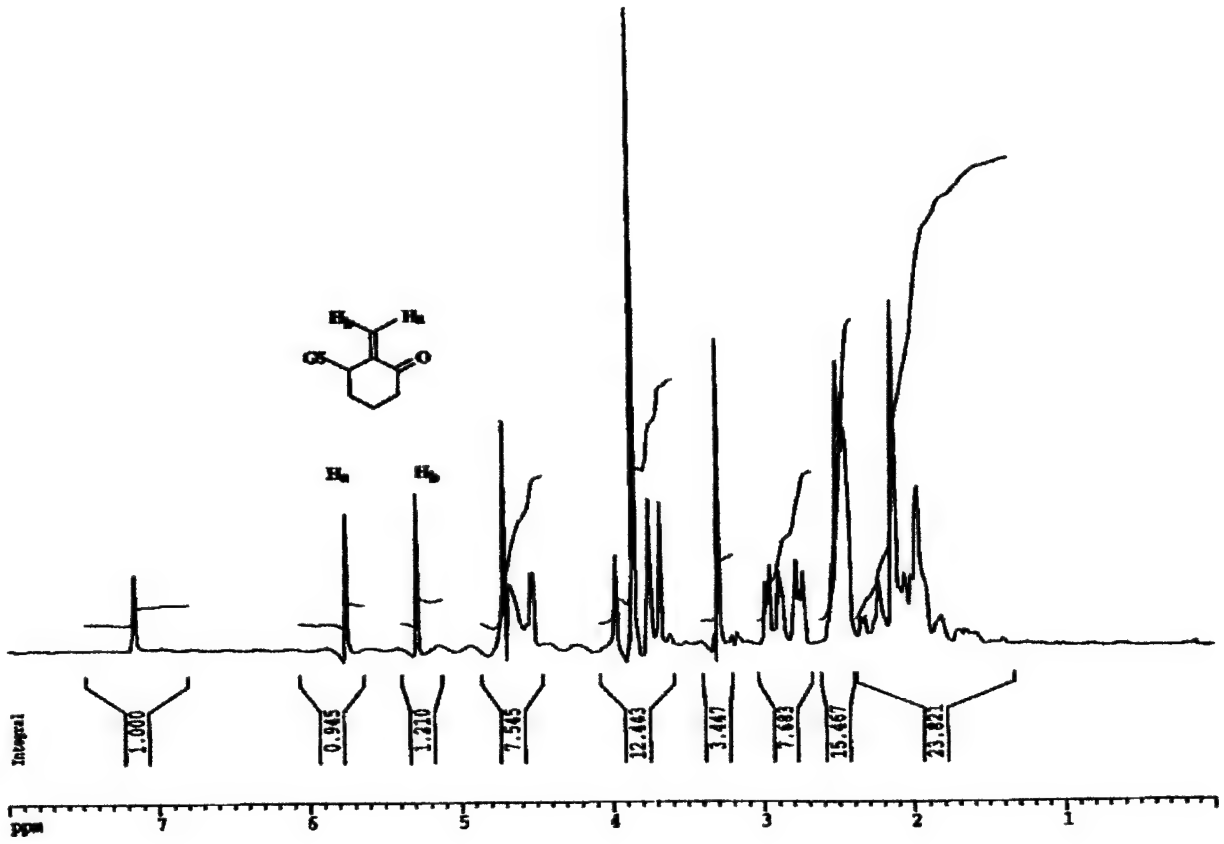


Fig. 4. *The 600 MHz ^{1}NMR spectrum of the putative exocyclic enone 2 corresponding to the 20.1 min peak in the HPLC elution profile of Figure 3.* The resonance at 7.16 ppm is due to the presence of adduct GSMC-6. The other resonances in the spectrum are those expected for S-substituted GSH derivatives[5].

The vinyl proton resonances at 5.76 and 5.29 ppm are characteristic of geminal vinylic hydrogens, and are consistent with published NMR spectra of several closely related exocyclic α-methylene cyclohexanones.[6]

C. Model building of GSMC-6 into the X-ray crystal structure of hGSTP1-1 (Hamilton et al.(2002), Appendix I). Working in collaboration with Ken Houk at the University of California, Los Angeles, we have recently completed model building studies designed to give greater insight into the mechanism by which hGSTP1-1 catalyzes the conversion of COMC-6 to the GSH adduct GSMC-6. In order to test the reliability of the AutoDock program, the GSH adduct of ethacrynic acid (GS-EA) was docked back into the binding pockets observed in the X-ray crystal structure and compared with the lowest energy binding mode of GS-EA using AutoDock, Fig. 5.

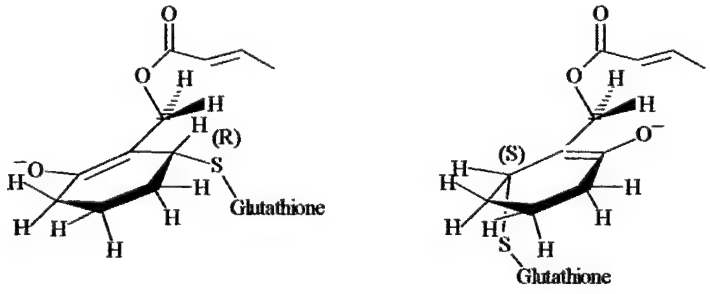
(a) (b)

Fig. 5. *Comparison of the structure of GS-EA derived from (a) the published X-ray structure of GS-EA in complex hGSTP1-1 and (b) docking of GS-EA into the active site of hGSTP1-1.*

The similarity of the modes of binding determined by x-ray crystallography versus computational modeling, indicates that AutoDock should reliably reproduce the binding modes of GSH conjugates in the active site.

Indeed, useful information was obtained from docking studies with the two diastereomeric enolates, which result from addition of GSH to the 2si-3re face or to the 2re-3si face of COMC-6, Chart 1.

Chart 1



The diastereomers were first constructed in Macromodel 7.1 and then docked into 3GSS. The structure of the glutathionyl function was taken from the crystal structure and kept rigid during docking, as GSH has about the same conformation bound to different *pi* class transferases.[3] Therefore, only three rotatable bonds were allowed to change: one between the crotonate ether oxygen and the neighboring methylene C, one between the methylene C and the adjacent ring C, and the one between the glutathionyl S and the adjacent ring C. The results are shown in Fig. 6 and agree best with an anti-addition-elimination

mechanism involving the formation of the 3-(R) enolate, given that this structure is about 1 Kcal more stable than that of the bound 3-(S) diastereomer.

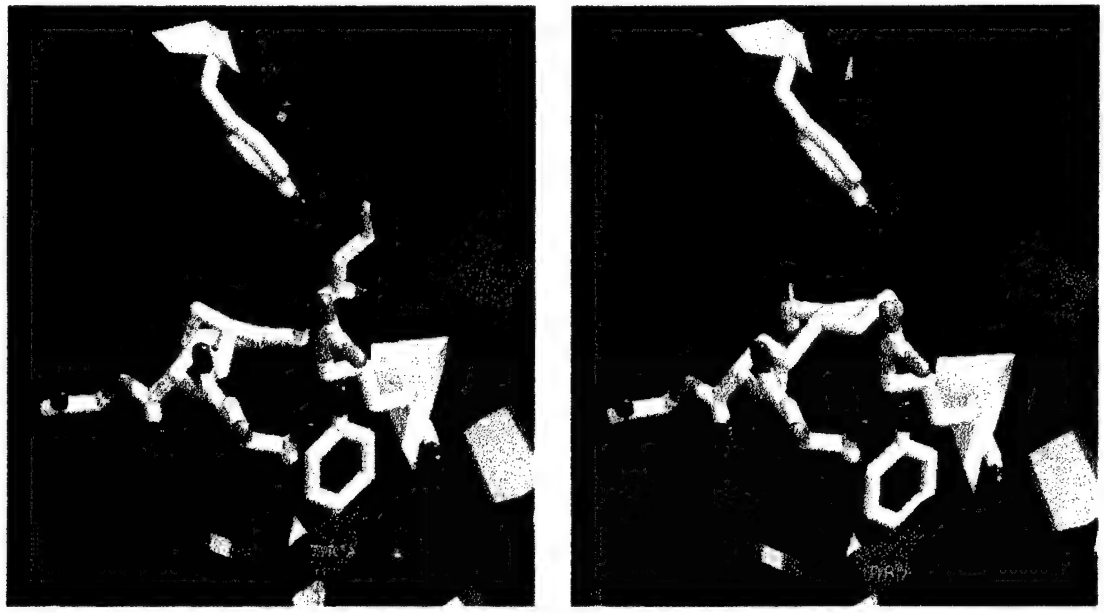


Figure. 6. *Lowest-energy binding modes of the (a) 3-R and (b) 3-S diastereomers of the enolates shown in Chart 1 bound to the active sites of hGSTP1-1.*

The docking studies are consistent with a mechanism in which Tyr 106 plays an electrophilic role in the catalyzed conversion of COMC-6 to the exocyclic enone **2**. This is similar to the mechanism previously proposed for the catalyzed addition of GSH to ethacrynic acid, as shown diagrammatically in Fig 7.

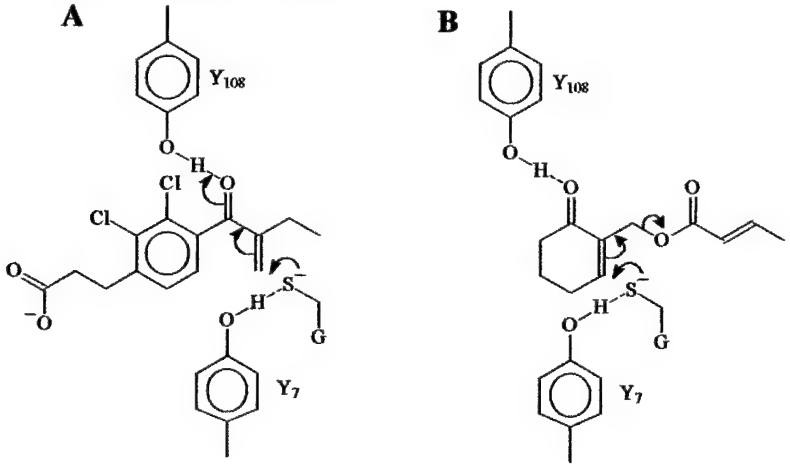
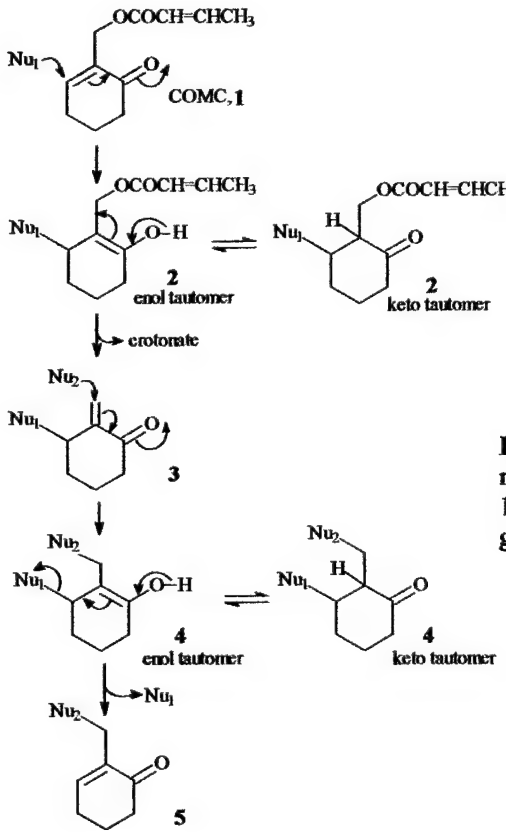


Fig. 7. *Proposed reaction mechanisms for glutathione transferase-catalyzed formation of the adducts between (A) GSH and ethacrynic acid* [3] *and (B) GSH and COMC-6.*

D. Mass spectral studies indicate thatCOMC-6 alkylates oligonucleotides *in vitro* [Zhang et al. (2002), Appendix 1]. In principle, the cytotoxicity of the substituted endocyclic enones could result from alkylation of DNA and/or proteins critical to cell function. Working in collaboration with Dan Fabre's laboratory at UMBC, the formation *in vitro* of a reactive glutathionyl exocyclic enone intermediate from COMC-6 and its ability to produce adducts with nucleic acids has been confirmed by direct mass spectrometric analysis of the reaction mixtures. Direct infusion electrospray ionization with an ion cyclotron resonance (ICR) analyzer was employed to identify the products of the *in vitro* reaction of COMC with GSH and with dinucleotides and oligonucleotides.

For a reaction mixture containing COMC-6 and GSH, protonated ions corresponding to the species shown in Scheme 3 (Nu_1=Nu_2=GSH) were detected in the positive ion mode (Figure 8).

Scheme 3



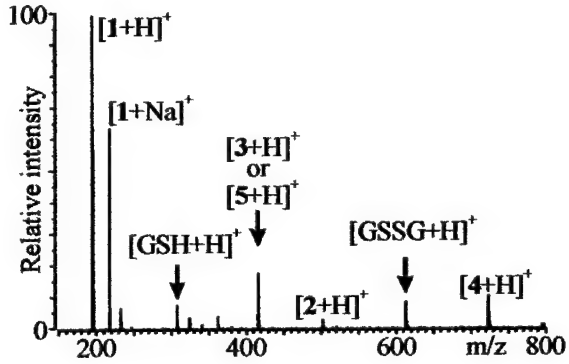


Fig 8. Positive ion mode ESI FT-MS spectrum of the product mixture initially composed of COMC and GSH in the presence of 1.2 units human glutathione-S-transferase, under the conditions given in the text.

Adduct **2**, either as the enol- or keto-tautomer, gave a weak, but recognizable, signal. This species presumably precedes the formation of the exocyclic enone **3.**

In order to assess the reactivity of COMC-6 with nucleic acids, COMC-6 and GSH were incubated in the presence of ribo-dinucleotides of different base composition. The products of the reaction with guanosylyl(3'-5')adenosine (GA) are shown in Figure 9. As expected species **2**, **3/5**, and **4** (with Nu_1=Nu_2=GSH) are readily recognizable in the

reaction mixture analyzed in positive ion mode (Figure 2A). However, only weak signals could be detected for protonated GA and GA-adducts.

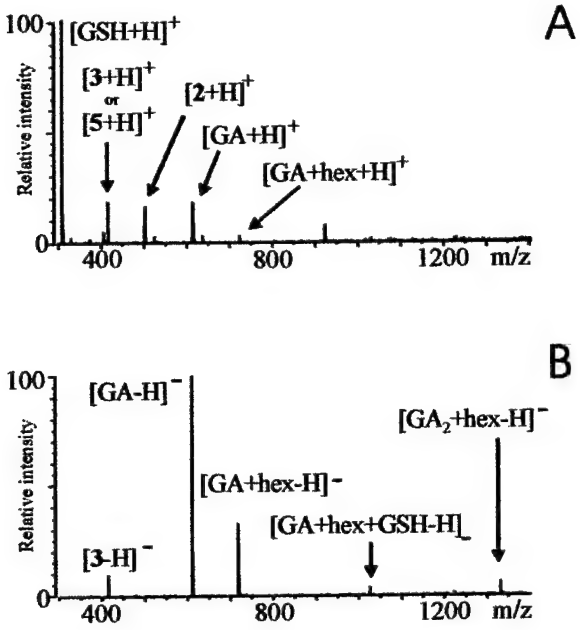


Figure 9. (A) Positive ion and (B) negative ion mode ESI FT-MS spectra of the product mixture initially composed of COMC, GA, and GSH. The abbreviation "hex" indicates the alkylating 2-methylene-2-cyclohexenone groups.

A more favorable signal-to-noise ratio was obtained in negative ion mode (Figure 9B), due to the negatively charged phosphate of the nucleotide function.

In order to identify the functional group(s) involved in the alkylation process, the dinucleotides, AA, GG, CC, UU, and TT (as 2'-deoxy-ribonucleotides) were tested with COMC. All substrates produced adducts with the exception of UU and TT. Thus, the likely sites of alkylation are the exocyclic amino groups of the nitrogenous bases, which are known to be favorable targets of electrophilic attack by genotoxic carcinogens.

The specificity of COMC was also evaluated with oligonucleotides of different length and base composition, either in their single stranded form or annealed with their complementary oligonucleotide to form a duplex. For example, adduct formation was readily observed when the single stranded DNA 16mer EY2 (ACG GAC CGG CGT ACG C) was treated with COMC (Figure 10A). The molecular mass was determined to be 4889.815 Da (4889.850 Da calculated from sequence for the ^{12}C monoisotopic species) for the starting 16mer, and 4997.901 Da (4997.907 Da from sequence) for the alkylated product. However, when the same reaction was carried out after annealing of EY2 with the complementary 16mer EY1 (GCG TAC GCC GGT CCG T), no alkylation was detected on either strand (Figure 10B). This finding suggests that the COMC function is attached to an exocyclic amino group, which is sterically protected by base pairing in the duplex oligonucleotide.

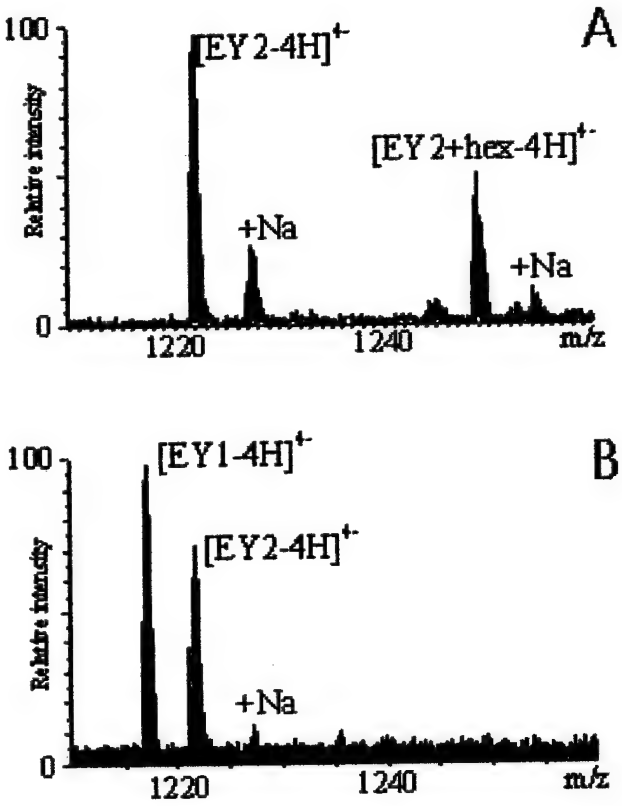
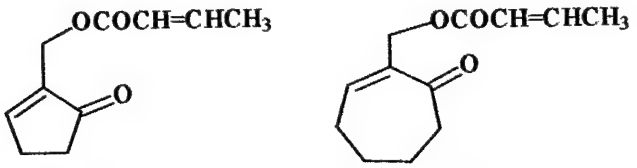


Fig. 10. Negative ion mode ESI FT-MS spectra of the reaction of COMC with (A) the 16mer oligonucleotide EY2 (ACG GAC CGG CGT ACG C) and with (B) a duplex formed after annealing with its complementary EY1 (GCG TAC GCC GGT CCG T). Only the region including the – 4 charge state is shown for better comparison.

In conclusion, the mass spectral studies clearly indicate that COMC-6 forms stable adducts *in vitro* with GSH and with nucleic acids. Adducts can form by either enzymatic or non-enzymatic pathways, following the addition-elimination mechanism shown in Scheme 3. This mechanism is strongly supported by the characterization of key intermediates along the reaction pathway.

E. The 5- and 7-membered ring homologues of COMC-6 are also toxic to B-16 *in vitro*, and COMC-6 displays similar toxicities toward both human colon adenocarcinoma HT29 and HT29 over-expressing the phosphoglycoprotein associated with some types of multidrug resistance [Joseph et al. (2002), Appendix I]. The respective 5- and 7-membered ring homologues of COMC-6 (Scheme 4) have been synthesized and also shown to undergo conjugate additions with GSH that involve intermediate exocyclic enones, as in Scheme 1.

Scheme 4



COMC-5 **COMC-7**

Therefore, these species should also exhibit antitumor activity. Indeed, all three COMCs are toxic to B16 murine melanoma *in vitro,* with IC_{50} values indicating an increase in potency with increasing ring size, Fig 11.

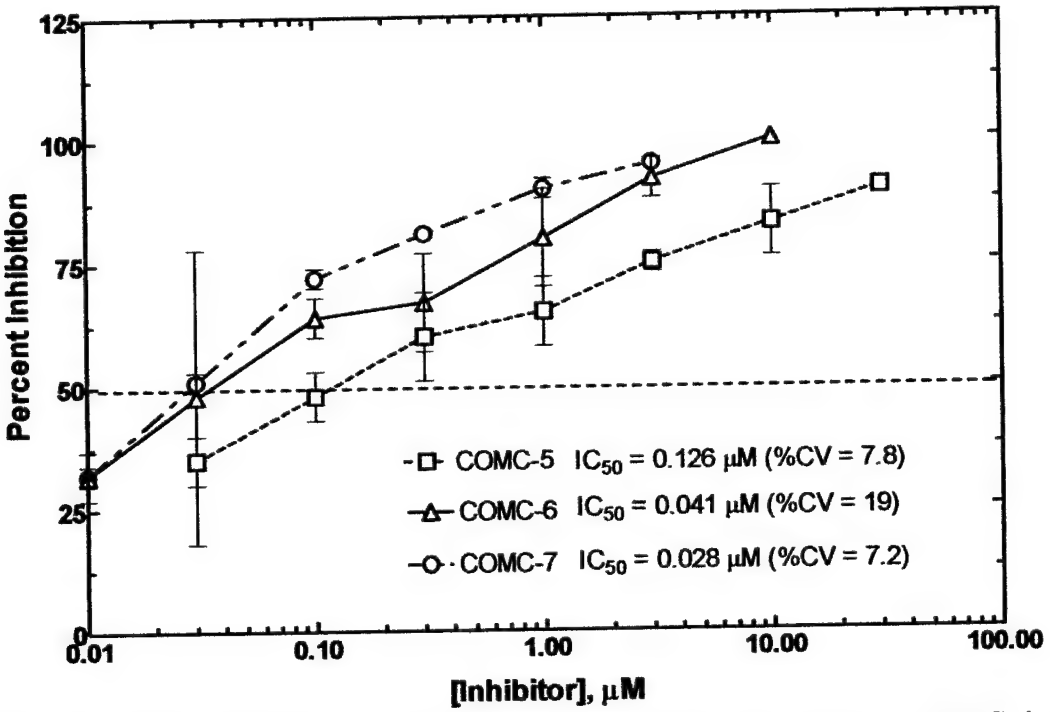


Figure 11. Growth inhibition of B16 cells in the presence of COMC-5, COMC-6 and COMC-7. Conditions/methods as in Fig. 1.

The similar shapes and parallel shift of the cytotoxicity curves suggest a similar mechanism of cytotoxicity for each compound. The IC_{50} values indicate an increase in potency with increasing ring size.

The cytotoxicity of COMC-6 was also examined in HT29(wt) human colon adenocarcinoma versus HT29 (MDR) cells that over express p-glycoprotein. This experiment was prompted by a previous observation that COMC derivatives display enhanced toxicity against certain types of drug-resistant neoplastic cells versus wild type cells. In the present study, the IC_{50} value for HT29 (wt) cells was 0.80 μM while that for HT29 (MDR) was 1.8 μM, a change of only 2-fold, Fig. 12

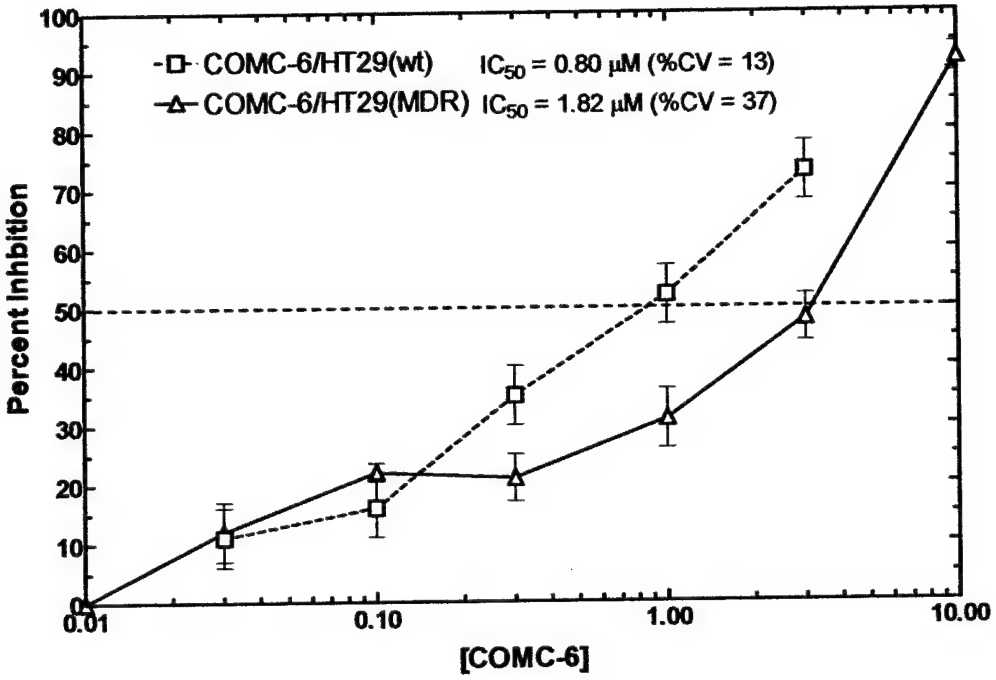


Fig. 12. Growth inhibition of HT29 (wt) versus HT29 (MDR) cells in the presence of COMC-6. Conditions/methods as in Fig. 1.

By comparison, a 17-fold difference in cytotoxicity of vincristine against these cell lines was noted in this laboratory: HT29 (wt), IC_{50} = 0.001 µM (%CV = .01); HT29 (MDR), IC_{50} = 0.017 µM (%CV = 17).

Thus, the intracellular cytotoxic species (presumably the exocyclic enone intermediate) appears to be a poor substrate for the MDR-associated p-glycoprotein, given the similar IC_{50} values of COMC-6 with HT29 (wt) versus HT29 (MDR). This finding might best be rationalized by the fact that the glutathionylated exocyclic enone is multiply charged, and that p-glycoprotein most readily transports uncharged, hydrophobic antitumor agents. This hypothesis might help to explain a previous observation that adriamycin-, aclarubicin-, and bleomycin-resistant sublines of murine lymphoblastoma L5178Y cells are no less sensitive to COTC than the parental cell line.

Key Research Accomplishments (during the third year)

- Found additional evidence that the glutathione (GSH) conjugate of COMC-6 is unlikely to account for the *in vitro* antitumor activity of COMC-6.
- Discovered that hGSTP1-1 efficiently catalyzes the conversion of COMC-6 to a reactive exocyclic enone of COMC-6.
- Demonstrated that the exocyclic enone can alkylate oligonucleotides *in vitro* and, therefore, alkylation of DNA *in vivo* is a possible basis of the antitumor activity of COMC-6.
- Synthesized and characterized the 5- and 7-membered ring homologues of COMC-6 (COMC-5, COMC-7) and demonstrated that COMC-7 is more potent to B16 tumor cells than COMC-6 *in vitro.*
- Demonstrated that COMC-6 displays similar toxicities to human colon HT29 versus HT29 over-expressing the phosphoglycoprotein responsible for some type of multidrug resistance.

Reportable Outcomes (years 1, 2 and 3)

Manuscripts in print or in press

1. Diana S. Hamilton and Donald J. Creighton (2001), "Using GSTP-1 to Generate Mechanism-based Competitive Inhibitors of the Anticancer Target Enzyme Glyoxalase I," Chemico-Biological Interactions, *133*, 355-359.

2. Avinash Kalsi, Malcolm J. Kavarana, Tianfenu Lu, Dale L. Whalen, Diana S. Hamilton, and Donald J. Creighton (2000), "Role of Hydrophobic Interactions in Binding S-(N-Aryl/Alkyl-N-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I," J. Med. Chem., *43* , 3981-3986.

3. C. Frederick M. Huntley, Diana S. Hamilton, Donald J. Creighton, and Bruce Ganem (2000), "Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor," Organic Lett., *2* , 3143-3144.

4. Diana. S. Hamilton, Zhebo Ding, Bruce Ganem and Donald J. Creighton (2002),"Glutathione S-Transferase- Catalyzed addition of Glutathione to COMC: A New Hypothesis for Antitumor Activity." Organic Lett., *4*, 1209-1212.

5. Quing. Zhang, Zhebo Ding, Donald J. Creighton, Bruce Ganem, and Daniel Fabris (2002),"Alkylation of Nucleic Acids by the Antitumor Agent COMC." Organic Lett., *4*, 1459-1462.

6. Erin Joseph, Julie L. Eiseman, Diana S. Hamilton, Heekyung Tak, Bruce Ganem, and Donald J. Creighton (2002a), "In Vitro Antitumor activity of endocyclic enone compounds: 2-crotonyloxymethyl-2-cyclohexenone (COMC-6),-cyclopentenone (COMC-5) or -cycloheptenone (COMC-7) in B16 murine melanoma and HT29 human colon adenocarcinoma," Proceedings of the American Association of Cancer Research, *43*, 386.

7. Erin Joseph, Julie L. Eiseman, Diana S. Hamilton, Haibo Wang, Heekyung Tak, Bruce Ganem, and Donald J. Creighton (2002b)," Molecular Basis of the Antitumor Activities of Crotonyloxymethyl-2-cyclohexenones."J. Med. Chem., in press.

8. Diana S. Hamilton, Zhebo Ding, Xiyun Zhang, Ina Hubatsch, Bengt Mannervik, Ken N. Houk, Bruce Ganem and Donald J. Creighton (2002), " Mechanism of the Glutathione Transferase-Catalyzed Addition of Glutathione to the Antitumor 2-Crotonyloxymethyl-2-cycloalkenones" Biochemistry, submitted.

Patent application:

1. B. Ganem, D.J. Creighton, D. Zheng, and D. S. Hamilton, "Enone Cancer Therapeutics" Tech No. AS202, filed 3/15/02 by Cornell University and the University of Maryland, Baltimore County.

Presentations at scientific meetings that describes work accomplished:

1. D.J. Creighton, "Tumor-selective Anticancer Strategies: Using GSTP1-1 to Generate Enediol Analogue Inhibitors of Glyoxalase I," GST 2000: International Conference on Glutathione Transferases, Uppsala University, Uppsala Sweden, 19-23 May 2000.

2. Diana S. Hamilton, Zhebo Ding, Bruce Ganem, and Donald J. Creighton, "Possible Mechanistic Basis of the Antitumor Activity of 2-Crotonyloxymethyl-2-cyclohexenone,"
34th ACS Middle Atlantic Regional Meeting (MARM), Towson University, Towson, Maryland, May 30 - June 1, 2001.

3. Diana S. Hamilton, Dan Fabre, Zhebo Ding, Bruce Ganem, and Donald J. Creighton, "GSTP1-1 Catalyzes the Addition of Glutathione to the Antitumor Agent 2-Crotonyloxymethyl-2-cyclohexenone Via a Highly Reactive Exocyclic Enone Intermediate," Gordon Research Conferences: Enzymes , Coenzymes and Metabolic Pathways, West Kingston, Rhode Island, July 22 - July 27, 2001.

4. D.J. Creighton, "Biochemical Basis of the Antitumor Activity of Endocyclic Eneones," University of Pittsburgh Cancer Center, April 19, 2001.

5. Erin Joseph, Julie L. Eiseman, Diana S. Hamilton, Heekyung Tak, Bruce Ganem, and Donald J. Creighton, "In Vitro Antitumor activity of endocyclic enone compounds: 2-crotonyloxymethyl-2-cyclohexenone (COMC-6),-cyclopentenone (COMC-5) or -cycloheptenone (COMC-7) in B16 murine melanoma and HT29 human colon adenocarcinoma," American Association for Cancer Research, 93 rd Annual Meeting, San Francisco California, April 6-10, 2002.

Conclusions

The are two central conclusions of this three-year study:

1. Compounds of the type YCON(OH)R, where Y equals different leaving groups and R equals different aryl substituents, are indeed very slow substrates for glutathione transferase (GST), producing as products GSH conjugates that are tight-binding inhibitors of the antitumor target enzyme glyoxalase I. This is an important "proof-of-concept" of our general hypothesis that the transferase can serve as a "drug factory" inside breast

tumor cells. However, but these compounds are unlikely to have practical significance, as they are such poor substrates for the transferase.

2. By far the most important conclusion of this work is based on our seminal observation that the COMCs are excellent substrates for human GST, producing as products powerful alkylating agents of DNA. Human glutathione transferase (hGSTP1-1) catalyzes the conversion of COMC-6 to the corresponding GSH adduct by a multi-step process involving the formation of a highly reactive exocyclic enone intermediate, which is the immediate product of enzyme catalysis. Conceivably, tumoricidal activity arises from the reaction of this electrophilic species with proteins and nucleic acids critical to cell survival. This conclusion is now well supported by our kinetic and mass spectrometry studies, as well as molecular docking experiments. The fact that GSTP1-1 catalyzes the formation of the cytotoxic exocyclic enone is of considerable interest for the control of breast cancer, as there are often large differences in GSTP1-1 activity between multidrug resistant breast tumors and peritumoral tissue that should contribute to the selective tumoricidal activities of compounds like COMCs. We are in the process of testing this hypothesis.

At the risk of sounding self-serving, more emphasis needs to be placed on compounds like the COMCs, whose activity is enhanced by the enzyme chemistry associated with multidrug resistance. This assertion is based on the fact that most fatalities among breast cancer patients with metastatic disease arise from recurrent tumors that exhibit the MDR phenotype.

References

1. Kavarana, M.J.; Kovaleva, E.G.; Creighton, D.J.; Wollman, M.B.; Eiseman, J.L. "Mechanism-Based Competitive Inhibitors of Glyoxalase I: Intracellular Delivery, In Vitro Antitumor Activities, and Stabilities in Human Serum and Mouse Serum." *J. Med. Chem.* **1999**, *42*, 221-228.

2. Aghil, O.; Bibby, M.C.; Carrington, S.J.; Doubic, J.; Douglas, K.T.; Phillips, R.M.; Shing, T.K.M. "Synthesis and Cytotoxicity of Shikimate Analogues. Structure:Activity Studies Based on 2-Crotonyloxymethyl-3R,4R,5R-trihydroxycyclohex-2-enone," *Anti-Cancer Drug Design* **1992,** *7*, 67-82.

3. Oakley, A. J.; Rossjohn, J.; Bello, M. L.; Caccuri, A. M., Federici, G.; Parker, M. W. "The Three-Dimensional Structure of the Human Pi Class Glutathione Transferase P1-1 in Complex with the Inhibitor Ethacrynic Acid and its Glutathione Conjugate." *Biochemistry* **1997**, *36*, 576-585.

4. Mannervik, B.; Danielson, U. H. "Glutathione Transferases-Structure and Catalytic Activity." *CRC Critical Reviews in Biochemistry* **1988**, *23*, 283-337.

5. Rabenstein, D. L.; Keire, D. A. "Nuclear Magnetic Resonance Spectroscopy of Glutathione." In *Coenzymes and Cofactors: Glutathione*; Dolphin, D.; Poulson, R.; Avramovic, O., Eds,; John Wiley, New York, 1989; Vol. 3, Part A, pp 67-101.

6. Tamura, R., Watabe, K., Ono, N., Yamamoto, Y. "Asymetric Synthesis of 3-Substituted 2-*exo*-Methylenealkanones by Addition-Elimination Reaction Using a Chiral Leaving Group and Organometallic Nucleophiles." *J. Org. Chem.* **1992**, *57*, 4895-4903.

APPENDIX I. Papers in press or in print-

related to Tasks 1 and 2

1. Diana S. Hamilton and Donald J. Creighton, "Using GSTP-1 to Generate Mechanism-based Competitive Inhibitors of the Anticancer Target Enzyme Glyoxalase I," Chemico-Biological Interactions, 133, 355-359, 2001.

2. Avinash Kalsi, Malcolm J. Kavarana, Tianfen Lu, Dale L. Whalen, Diana S. Hamilton, and Donald J. Creighton, "Role of Hydrophobic Interactions in Binding S-(N-Aryl/Alkyl-N-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I," J. Med. Chem., Vol. 43, No. 21, 3981-3986, 2000.

related to Task 3,4,and 5

3. C. Frederick M. Huntley, Diana S. Hamilton, Donald J. Creighton, and Bruce Ganem (2000), "Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor," Organic Lett., *2*, 3143-3144.

4. Diana. S. Hamilton, Zhebo Ding, Bruce Ganem and Donald J. Creighton (2002),"Glutathione S-Transferase- Catalyzed addition of Glutathione to COMC: A New Hypothesis for Antitumor Activity." Organic Lett., *4*, 1209-1212.

5. Quing. Zhang, Zhebo Ding, Donald J. Creighton, Bruce Ganem, and Daniel Fabris (2002),"Alkylation of Nucleic Acids by the Antitumor Agent COMC." Organic Lett., *4*, 1459-1462.

6. Erin Joseph, Julie L. Eiseman, Diana S. Hamilton, Heekyung Tak, Bruce Ganem, and Donald J. Creighton (2002a), "In Vitro Antitumor activity of endocyclic enone compounds: 2-crotonyloxymethyl-2-cyclohexenone (COMC-6),-cyclopentenone (COMC-5) or -cycloheptenone (COMC-7) in B16 murine melanoma and HT29 human colon adenocarcinoma," Proceedings of the American Association of Cancer Research, *43*, 386.

7. Erin Joseph, Julie L. Eiseman, Diana S. Hamilton, Haibo Wang, Heekyung Tak, Bruce Ganem, and Donald J. Creighton (2002b)," Molecular Basis of the Antitumor Activities of Crotonyloxymethyl-2-cyclohexenones."J. Med. Chem., in press.

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Using GSTP1-1 to generate mechanism-based competitive inhibitors of the anticancer target enzyme glyoxalase I

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Abstract

Substrates for GSTP1-1 that produce as products inhibitors of the methylglyoxal detoxifying enzyme glyoxalase I might exhibit selective tumoricidal activity against multidrug resistant (MDR) tumors, as the transferase is often overexpressed at high levels in these tumors. In support of the feasibility of this strategy, we have discovered that selected *N*-hydroxy carbamoyl esters ($ClC_6H_4OC(O)N(OH)R$, where $R = ZC_6H_4$(Z = H, Cl, Br)) are slow substrates for human placental GSTP1-1, producing as products powerful, mechanism-based competitive inhibitors of human glyoxalase I (GSC(O)N(OH)R, where GS = glutathionyl). In contrast, the transferase did not exhibit detectable activity with carbamoyl esters in which R = alkyl.

Keywords: Carbamoyl esters; Glyoxalase I inhibitors; GST substrates; MDR tumors

The glyoxalase enzyme system has been the focus of much recent attention as a potential target for antitumor drug development [1,2]. This enzyme system, composed of the isomerase glyoxalase I (GlxI) and the thioester hydrolase glyoxalase II, promotes the glutathione(GSH)-dependent conversion of cytotoxic methylglyoxal to D-lactate [3,4]. We recently demonstrated that both S-(*N*-aryl/alkyl *N*-hydroxycarbamoyl)glutathione derivatives (**1**) are powerful competitive inhibitors of human GlxI with K_i values in the nanomolar concentration range [5–7].

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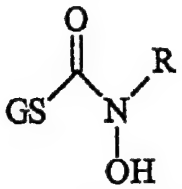
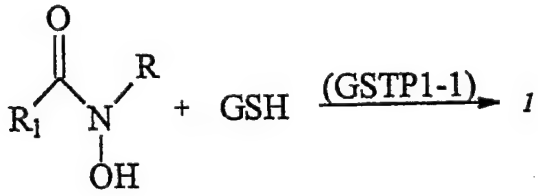


1 (R = C_6H_4, C_6H_4Cl, C_6H_4Br, $(CH_2)_nCH_3$ (where n = 0 - 6))

Tight binding appears to result from the fact that these compounds are stable intermediate analogues of the enediolate intermediate that forms along the reaction coordinate of the enzyme [8]. The diethyl ester prodrugs of the *N*-aryl derivatives inhibit the growth of both murine and human tumors in vitro [9] and in vivo [10]. Tumor toxicity appears to be due to inhibition of intracellular GlxI, which results in elevated concentrations of methylglyoxal [9]. Taken together, these results emphasize the potential importance of GlxI as a novel antitumor target.

Like the glyoxalase enzymes, the glutathione *S*-transferases (GSTs) play an important detoxification role in cells by catalyzing the addition of GSH to different electrophilic xenobiotics [11,12]. Among the many different members of the GST family of enzymes, the GSTP1-1 isozyme is of particular interest from a cancer control perspective. This isozyme is significantly elevated in solid human tumors of the colon, lung and particularly breast with only a few tumors showing abnormally low levels of this isozyme [13]. Tumors exhibiting the multidrug resistance (MDR) phenotype can have GSTP1-1 activities that are 15-fold higher than normal [14]. Several different laboratories are in the process of developing specific inhibitors of GSTP1-1 in order to overcome drug resistance [15].

In contrast, we aim to take advantage of the high levels of GSTP1-1 activity in MDR tumor cells by using the transferase to generate cytotoxic enediol analogues of the GlxI reaction in situ. In order to test this hypothesis, carbamoyl esters **2** were synthesized using methods previously described by this laboratory [5–7] and evaluated as substrates for human placental GSTP1-1, Eq. (1).



(1)

2 (R_1 = p-ClC_6H_4O, CH_3CH_2S)

The transferase was purchased from Sigma Chemical Co. and salts and free GSH were removed by ultrafiltration. The carbamoyl esters (**2**) were separately incubated in the presence and in the absence of 2 U of GSTP1-1 for 69 h under the condition

given in the legend to Fig. 1. The solvent was removed in vacuo and the residue resolved on a reverse-phase C 18 column using a methanol/water mixture, containing 0.25% acetic acid, as a running solvent. The amount of GSH conjugate formed was determined from a comparison of the integrated intensity of the peak, corresponding to that of authentic GSH conjugate, with standard curves of peak area versus concentration. The activities of the different substrates with the transferase were computed from the amount of conjugate formed in the presence of enzyme minus that formed in the absence of enzyme.

The results of these preliminary studies suggest a tentative structure-activity correlation, namely, that carbamoyl esters in which both R and R_1, are aromatic functions are slow substrates for GSTP1-1, Fig. 1. This correlation is based on the observation that no significant activity was found with any of the *N*-alkyl carbamoyl esters having p-chlorophenol as a leaving group. Moreover, no activity was

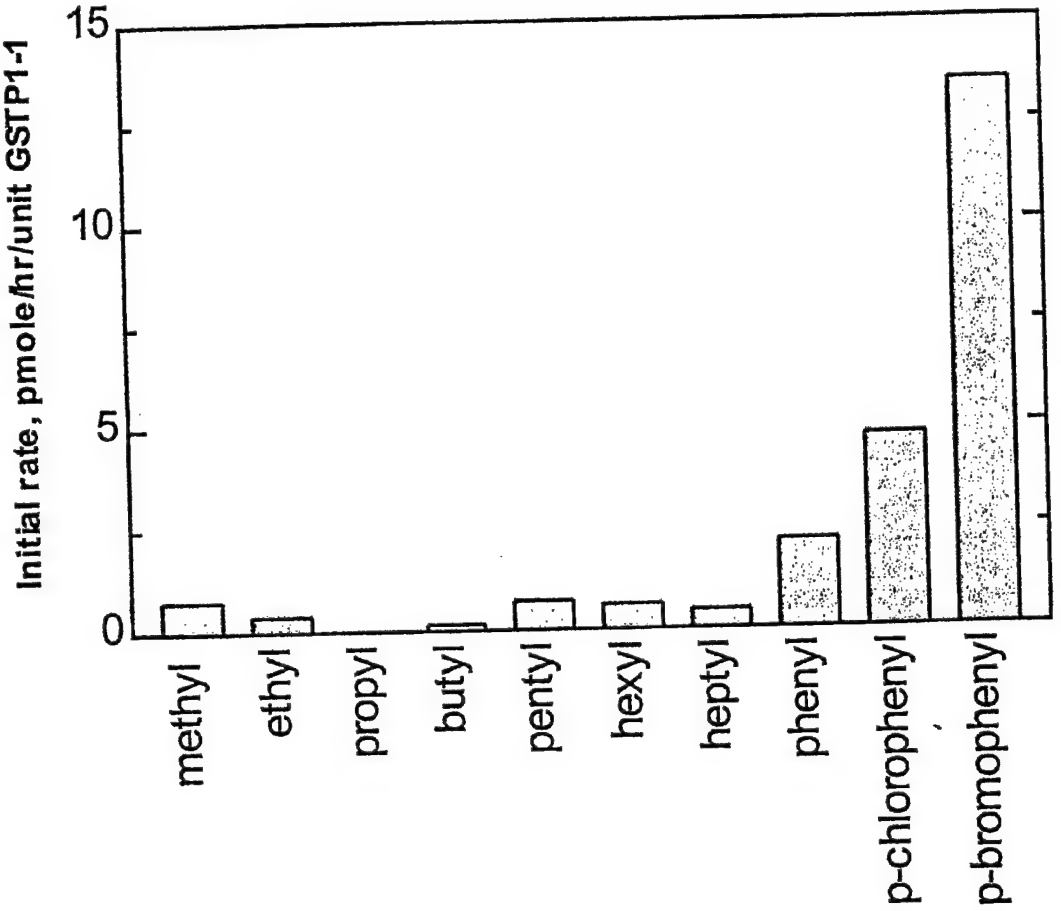


Fig. 1. Comparison of the activities of human GSTP1-1 with the carbamoyl esters shown in Eq. (1), where R_1 = p-chlorophenol. Conditions: Sodium Phosphate buffer, pH 7.5, containing 30% (v/v) ethanol and 0.05 mM ethylenediamine tetraacetate; 1 mM GSH, 0.4 mM carbamoyl ester, human placental GSTP1-1 (2.5 U using 2,4dinitrochlorobenzene as substrate), 22°C.

detected with the *N*-p-chlorophenyl derivative having ethanthiol as a leaving group. However, significant activity was found for the *N*-aryl derivatives in which p-chlorophenol is the leaving group. Apparently, the diaryl carbamoyl esters bind to the transferase in such a way as to allow significant transition state stabilization by the active site residues. We are currently attempting to improve the substrate properties of the carbamoyl esters by optimizing substrate structure using molecular docking methods with the known x-ray structure of human GSTP1-1.

This is the first demonstration that carbamoyl esters can be designed to serve as substrates for GSTP1-1, giving as products enediol analogue inhibitors of GlxI. This is the basis of a potentially novel tumor-selective anticancer strategy.

Acknowledgements

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References

[1] P.J. Thornalley, Glutathione-dependent detoxification of alpha-oxoaldehydes by the glyoxalase system: involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors, Chem. Biol. Interact. 111–112 (1998) 137–151.

[2] D.J. Creighton, D.S. Hamilton, M.J. Kavarana, E.M. Sharkey, J.L. Eiseman, Glyoxalase enzyme system as a potential target for antitumor drug development, Drugs of the Future 25 (2000) 385–392.

[3] D.L. Vander Jagt, The glyoxalase system, in: D. Dolphin, P.R. Poulson, O. Avramovic (Eds.), Coenzymes and Cofactors: Glutathione, vol. 3, Wiley, New York, 1989, pp. 597–641.

[4] D.J. Creighton, T. Pourmotabbed, Glutathione-dependent aldehyde oxidation reactions, in: J.F. Liebman, A. Greenberg (Eds.), Molecular Structure and Energetics: Principles of Enzyme Activity, vol. 9, VCH Publishers, New York, 1988, pp. 353–386.

[5] D.S. Hamilton, D.J. Creighton, Inhibition of glyoxalase I by the enediol mimic S-(N-hydroxy-N-methylcarbamoyl)glutathione: the possible basis of a tumor-selective anticancer strategy, J. Biol. Chem. 267 (1992) 24933–24936.

[6] N.S.R.K. Murthy, T. Bakeris, M.J. Kavarana, D.S. Hamilton, Y. Lan, D.J. Creighton, S-(N-Aryl-N-hydroxycarbamoyl)glutathione derivatives are tight-binding inhibitors of glyoxalase I and slow substrates for glyoxalase II, J. Med. Chem. 37 (1994) 2161–2166.

[7] Kalsi, M.J. Kavarana, T. Lu, D.L. Whalen, D.S. Hamilton, D.J.Creighton, Role of hydrophobic interactions in binding S-(N-aryl/alkyl-N-hydroxycarbamoyl)glutathiones to the active site of the anti-tumor target enzyme glyoxalase I, J. Med. Chem. 43 (2000) 3981–3986.

[8] A.D. Cameron, M. Ridderstrom, B. Olin, M.J. Kavarana, D.J. Creighton, B. Mannervik, Reaction mechanism of glyoxalase I explored by an x-ray crystallographic analysis of the human enzyme in complex with a transition state analogue, Biochemistry 38 (1999) 13480–13490.

[9] M.J. Kavarana, E.G. Kovaleva, D.J. Creighton, M.B. Wollman, J.L. Eiseman, Mechanism-based competitive inhibitors of glyoxalase I: intracellular delivery, in vitro antitumor activities, and stabilities in human serum and mouse serum, J. Med. Chem. 42 (1999) 221–228.

[10] E.M. Sharkey, H.B. O'Neill, M.J. Kavarana, H. Wang, D.J. Creighton, D.L. Sentz, J.L. Eiseman, Pharmacokinetics and anti-tumor properties in tumor-bearing mice of an enediol analogue inhibitor of glyoxalase I, Cancer Chemother. and Pharmacol. 46 (2000) 2, 156–166.

[11] B. Mannervik, The isozymes of glutathione transferase, Adv. Enzymol. 57 (1985) 357–417.
[12] R.N. Armsrtong, Glutathione S-transferases: structure and mechanism of an archetypical detoxifying enzyme, Adv. Enzymol. 69 (1994) 1–44.
[13] J.A. Montali, J.B. Wheatley, D.E. Schmidt, Jr, Expression of GST in normal and malignant tissues, Cellular Pharmacol. 2 (1995) 241–247.
[14] C.R. Wolf, C.J. Wareing, S.M. Black, J.D. Hayes, Glutathione S-transferases in resistance to chemotherapeutic drugs, in: J.D. Hayes, C.B. Pickett, T.J. Mantle (Eds.). Glutathione S-Transferases and Drug Resistance, Taylor and Francis, London, 1990.
[15] M. Lyttle, M.D. Hocker, H.C. Hui, C.G. Caldwell, D.T. Aaron, A. EngquistGoldstein, J.E. Flatgaard, K.E. Bauer, Isozyme specific glutathione-transferase inhibitors: design and synthesis, J. Med. Chem. 37 (1994) 189–194.

Role of Hydrophobic Interactions in Binding *S*-(*N*-Aryl/Alkyl-*N*-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I

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Hydrophobic interactions play an important role in binding *S*-(*N*-aryl/alkyl-*N*-hydroxycarbamoyl)glutathiones to the active sites of human, yeast, and *Pseudomonas putida* glyoxalase I, as the log K_i values for these mechanism-based competitive inhibitors decrease linearly with increasing values of the hydrophobicity constants (π) of the *N*-aryl/alkyl substituents. Hydrophobic interactions also help to optimize polar interactions between the enzyme and the glutathione derivatives, given that the K_i value for *S*-(*N*-hydroxycarbamoyl)glutathione (π = 0) with the human enzyme is 35-fold larger than the interpolated value for this compound obtained from the log K_i versus π plot. Computational studies, in combination with published X-ray crystallographic measurements, indicate that human glyoxalase I binds the syn-conformer of *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathiones in which the *N*-aryl substituents are in their lowest-energy conformations. These studies provide both an experimental and a conceptual framework for developing better inhibitors of this antitumor target enzyme.

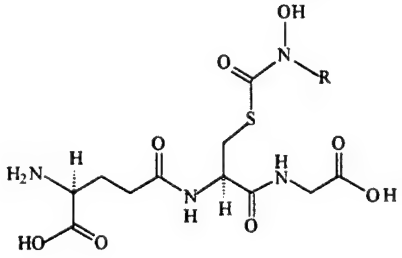
Introduction

The glyoxalase enzyme system has been the focus of much recent attention as a potential target for antitumor drug development.[1,2] This enzyme system, composed of the isomerase glyoxalase I (GlxI) and the thioester hydrolase glyoxalase II (GlxII), promotes the glutathione (GSH)-dependent conversion of cytotoxic methylglyoxal to D-lactate.[3,4] The toxicity of methylglyoxal, a byproduct of normal cellular metabolism,[5] appears to arise from its ability to cross-link proteins and to form adducts with DNA.[6,7] We recently demonstrated that selected *S*-(*N*-aryl-*N*-hydroxycarbamoyl)-glutathiones **1a**−**c** (Chart 1, Table 1) are powerful competitive inhibitors of human GlxI[8,9] and that the [*glycyl,glutamyl*]diethyl ester prodrugs inhibit the growth of both murine and human tumors in vitro[10] and in vivo.[11] Tumor toxicity appears to be due to inhibition of intracellular GlxI, which results in elevated levels of methylglyoxal.[10] Thus, GlxI appears to be a potentially important antitumor target.

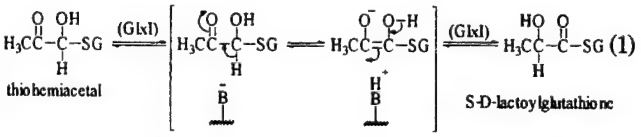
Understanding the nature of the binding interaction between the *N*-aryl-*N*-hydroxycarbamoyl esters of GSH and the active site of GlxI is clearly important, to provide a better basis for inhibitor design. Binding appears to result from two conceptually separate and distinct phenomena. First, the *N*-hydroxycarbamoyl ester function contributes to binding by modeling the stereoelectronic features of the tightly bound enediol intermediate and/or flanking transition states that form from the GSH−methylglyoxal−thiohemiacetal substrate, eq 1.[9]

Second, the *N*-aryl substituent also makes a major contribution to binding by interacting with a hydrophobic pocket in the active site, as indicated by the increase in binding affinity with increasing hydrophobicity of the *N*-aryl substituent. The recently determined high-resolution X-ray crystal structure of the homodimeric human enzyme in complex with enediol analogue **1d** confirms the presence of a hydrophobic binding pocket in the active site.[12] In the structure, a catalytically essential active site Zn^{2+} directly coordinates both oxygen atoms of the syn-conformation of the *N*-hydroxy-

Chart 1. Structures of *S*-(*N*-Aryl-*N*-hydroxycarbamoyl)glutathiones **1a**−**d** and *S*-(*N*-Alkyl-*N*-hydroxycarbamoyl)glutathiones **2a**−**h**



compd	R	compd	R	compd	R
1a	C_6H_5	*2a*	H	*2e*	$(CH_2)_3CH_3$
1b	4-C_6H_4Cl	*2b*	CH_3	*2f*	$(CH_2)_4CH_3$
1c	4-C_6H_4Br	*2c*	CH_2CH_3	*2g*	$(CH_2)_5CH_3$
1d	4-C_6H_4I	*2d*	$(CH_2)_2CH_3$	*2h*	$(CH_2)_6CH_3$



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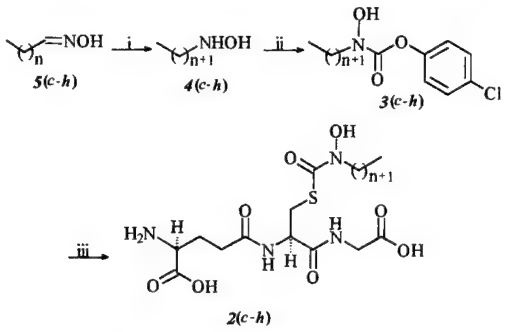
Scheme 1. Synthesis of *S*-(*N*-Alkyl-*N*-hydroxycarbamoyl)glutathiones **2c**–**h**[a]



c: n = 0, *d*: n = 1, *e*: n = 2, *f*: n = 3, *g*: n = 4, *h*: n = 5

[a] Reagents and conditions: (i) $(C_2H_5)_3SiH$; (ii) 4-chlorophenyl chloroformate; (iii) GSH/EtOH–H_2O, pH 9.

carbamoyl ester function, and the *N*-aryl substituent occupies a hydrophobic pocket composed of Phe 67A, Phe 62A, Leu 69A, Cys 60A, Phe 71A, Ile 88A, Leu 92A, Leu 174B, Leu 160B, Phe 162B, and Met 157B.

As part of a research program aimed at developing highly specific, tight-binding inhibitors of human GlxI, we have probed the dimensions and properties of the hydrophobic binding pocket using both experimental and computational methods. Essential to the conclusions of this study is a comparison of the inhibition constants of the *N*-aryl-*N*-hydroxycarbamoyl esters of GSH (**1a**–**c**) versus the homologous series of *N*-alkyl-*N*-hydroxycarbamoyl esters of GSH (**2a**–**h**) shown in Chart 1.

Chemistry and Enzymology

The *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathione derivatives **1a**–**c** were prepared by reaction of GSH with the 4-chlorophenyl esters of the corresponding *N*-aryl-*N*-hydroxycarbamates, as previously described by this laboratory.[9] *S*-(*N*-Hydroxycarbamoyl)glutathione (**2a**) was prepared by an acyl-interchange reaction between *N*-hydroxycarbamate 4-chlorophenyl ester (**3a**) and GSH. The acylating reagent (**3a**) was prepared by reacting hydroxylamine with 4-chlorophenyl chloroformate. *S*-(*N*-Hydroxy-*N*-methylcarbamoyl)glutathione (**2b**) was prepared by a published method from this laboratory.[8] The *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones **2c**–**h** were synthesized as outlined in Scheme 1. The 4-chlorophenyl esters of the *N*-alkyl-*N*-hydroxycarbamates **3c**–**h** were prepared using a modification of the general method of Wu and Sun.[13] In a one-pot reaction mixture, triethylsilane was first used to reduce the alkyl oximes **5c**–**h** to the corresponding hydroxylamines **4c**–**h**, followed by reaction with 4-chlorophenyl chloroformate to give **3c**–**h**. Reaction of GSH with **3c**–**h** gave crude preparations of **2c**–**h**, which were purified to apparent homogeneity by differential precipitation or reverse-phase HPLC. NMR spectral assignments were based on comparisons with previously published NMR studies of GSH and its derivatives.[14]

Human erythrocyte GlxI was purified to homogeneity from outdated human blood by a published procedure.[15] *Pseudomonas putida* GlxI was purified from *Escherichia coli* BL21(DE3) transformed with pBTac1/GlxI,[16] using methods described elsewhere.[17]

Table 1. Inhibition Constants (K_i's) of *S*-(*N*-Aryl-*N*-hydroxycarbamoyl)glutathiones with Human Erythrocyte, Yeast, and *P. putida* GlxI[a]

		K_i (μM)		
compd	π[b]	human[c]	yeast[c]	*P. putida*
2b	0.54	1.7 ± 0.1	68 ± 5	86 ± 9
1a	2.13	0.16 ± 0.1	11 ± 1	28 ± 3
1b	3.04	0.046 ± 0.004	3.6 ± 0.3	16 ± 1
1c	3.25	0.014 ± 0.001	1.2 ± 0.2	10 ± 2

[a] Phosphate buffer (50 mM, pH 7), 25 °C. [b] Hansch hydrophobicity constants for R-substituents obtained from ref 20. [c] From ref 9.

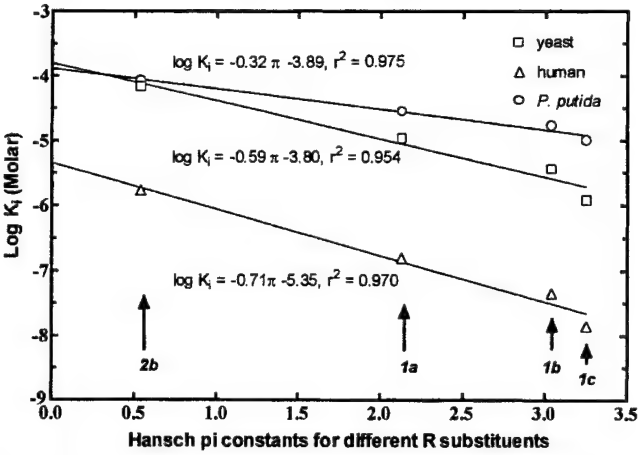


Figure 1. Log plot of competitive inhibition constants (K_i's) versus the Hansch hydrophobicity constants of the R-substituents for *S*-(*N*-methyl-*N*-hydroxycarbamoyl)glutathione (**2b**) and different *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathiones **1a**–**c** with human, yeast, and *P. putida* GlxI. The data for human and yeast GlxI were taken from ref 9.

Results and Discussion

The presence of a hydrophobic binding pocket in the active site of yeast GlxI was first suggested by Vince and co-workers, on the basis of the progressive decrease in the inhibition constants of simple *S*-aryl and *S*-alkyl GSH derivatives with increasing hydrophobicity of the *S*-substituent.[18]

Role of Hydrophobicity in Inhibitor Binding. Indeed, the binding affinities of the enediol analogues **2b** and **1a**–**c** for human, yeast, and *P. putida* GlxI increase with increasing hydrophobicity of the *N*-substituent (Table 1), indicated by the inverse relationship between log K_i and the hydrophobicity constants (π) of the *N*-methyl and *N*-aryl functions, Figure 1.[19] The π constant is defined as the log of the *n*-octanol/water partition ratio for the *N*-aryl function, calculated according to the method of Hansch.[20] The slopes of the lines through the data measure the change in the free energy of transfer of the enediol analogues from aqueous buffer at pH 7 to the active sites of the enzymes *relative* to the change in the free energy of transfer of the analogues between buffer and *n*-octanol. Thus, the slopes for the yeast and human enzymes can be interpreted to indicate that the hydrophobicities of the respective binding pockets are about 60% and 70% that of *n*-octanol. The shallower slope for *P. putida* GlxI indicates a hydrophobicity about 30% that of *n*-octanol.

Polar/charged residues that are *not* present in the hydrophobic pockets of the human and yeast enzymes can explain the smaller apparent hydrophobicity of the

Table 2. Inhibition Constants (K_i's) of *S*-(*N*-Alkyl-*N*-hydroxycarbamoyl)glutathiones with Human Erythrocyte GlxI[a]

compd	π^b	K_i (μM)	compd	π^b	K_i (μM)
2a	0	183 ± 50	**2f**	2.70	0.17 ± 0.08
2c	1.08	1.18 ± 0.07	**2g**	3.24	0.016 ± 004
2d	1.62	0.80 ± 0.5	**2h**	3.78	0.018 ± 0.011
2e	2.16	0.18 ± 0.09			

[a] Phosphate buffer (100 mM, pH 7), 25 °C. [b] Hansch hydrophobicity constants for R-substituents calculated according to ref 20.

binding pocket in the bacterial enzyme. While there is no reported structural data for the yeast and bacterial enzymes, sequence comparisons with the human enzyme suggest the presence of analogous hydrophobic pockets in the active sites of the yeast and bacterial enzymes. In the human enzyme, Leu 92A and Ile 88A are 2 of 11 residues that compose the hydrophobic pocket identified in the X-ray crystal structure.[21] These two residues are replaced, respectively, by Lys and His residues in the sequence of amino acids that comprise the apparent hydrophobic pocket in the bacterial enzyme, presumably resulting in a lower overall hydrophobicity. In contrast, Leu 92A and Ile 88A are conservatively replaced by two Phe residues in the yeast enzyme, consistent with the similar observed hydrophobicities for the yeast and human enzymes.

The binding affinities to human GlxI of a homologous series of *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones **2a**–**h** (Chart 1) were determined for comparison with those of the *N*-aryl derivatives, Table 2. Initially, we anticipated that the slope of the log K_i versus π plot would be shallower than that observed with the *N*-aryl derivatives (Figure 1), because of the increasing conformational flexibility of the *N*-alkyl function proceeding from *N*-methyl to *N*-heptyl. Conformational flexibility (entropy) should negatively impact binding affinity, if the active site binds only a limited subset of *N*-alkyl conformers that exist in bulk solvent. By one estimate, for each degree of rotational freedom lost upon binding of a ligand to a protein, there is roughly a 9-fold decrease in binding affinity.[22] This effect will be less important proceeding from *N*-methyl to the *N*-aryl derivatives, as there is no systematic change in conformational flexibility of the *N*-substituent. However, contrary to expectation, the slopes for the two classes of compounds are identical within experimental error, Figure 2. This suggests either that there is little change in conformational flexibility of the *N*-alkyl derivatives upon binding to the enzyme *or* that there are compensating favorable enthalpic and/or entropic terms (due to solvent/protein reorganization), which are not reflected in the binding affinities of the *N*-aryl derivatives to the active site. *In either case, conformational flexibility appears not to adversely affect the binding affinity of the N-alkyl enediol analogues with the active site of GlxI.*

Role of Polar Interactions in Inhibitor Binding. The intercept values on the log K_i axis of Figure 1 reflect the contribution of polar interactions to binding affinity. These values are the hypothetical inhibition constants for an enediol analogue in which the *N*-aryl function is replaced by *N*-H ($\pi = 0$), a functionality that cannot interact with the enzyme hydrophobically. The difference in the intercept values indicates that polar interactions make a 25-fold greater contribution to binding

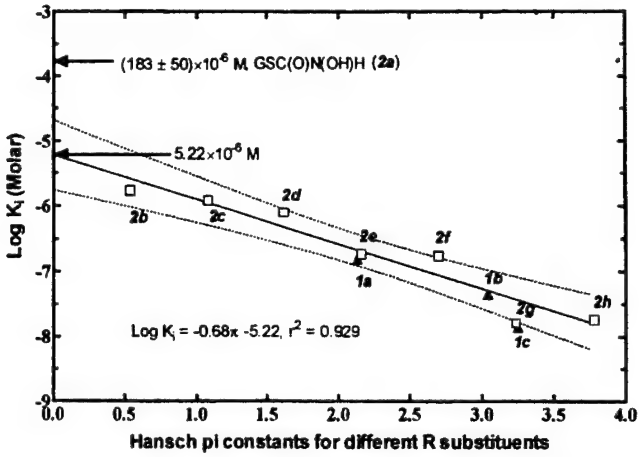


Figure 2. Log plot of competitive inhibition constants (K_i's) versus the Hansch hydrophobicity constants of the R-substituents for *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones **2b**–**h** (□) and *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathiones **1a**–**c** (▲) with human GlxI. The solid line is the result of linear regression analysis of the data for *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones. The dashed lines demarcate the 95% confidence interval. Also shown is the experimentally determined inhibition constant for *S*-(*N*-hydroxycarbamoyl)glutathione (**2a**).

affinity for the human enzyme than for the yeast and bacterial enzymes. Sequence comparisons indicate that one of the ligands (Gln 33) to the active site Zn^{2+} of the human enzyme is replaced by His in the yeast and bacterial enzymes.[21] Conceivably, this might indirectly influence the stability of the direct coordination interaction between the Zn^{2+} and the *N*-hydroxycarbamoyl ester function of the bound enediol analogues. The difference in binding affinities is less easily explained by differential interactions with the glutathionyl moiety, as the active site residues that interact with the glutathionyl backbone of the bound inhibitors are strictly conserved among the enzymes from the three different biological sources.[21]

Of substantial interest is the observation that the experimentally measured value of the inhibition constant for *S*-(*N*-hydroxycarbamoyl)glutathione (**2a**, K_i = 183 μM) is 35-fold larger than the extrapolated value of 5.2 μM for this compound obtained from the intercept on the log K_i axis of the hydrophobicity plot of Figure 2. This strongly indicates that occupancy of the hydrophobic binding pocket somehow optimizes polar interactions between the enzyme and the bound ligand, perhaps by promoting alignments to the active site zinc ion. *This is the first evidence for a cooperative relationship between the polar and hydrophobic interactions associated with binding an enediol analogue to the active site of GlxI.*

Computational Studies. The binding of the *N*-aryl enediol analogues to the active site could be adversely affected by the presence of steric restrictions in the hydrophobic binding pocket. For example, the X-ray crystal structure of the binary GlxI·**1d** complex shows an orthogonal relationship between the plane of the phenyl ring and the plane of the *N*-hydroxycarbamoyl ester function.[12] The question of whether this might reflect steric bumping between the phenyl ring and active site residues was addressed using computational methods.

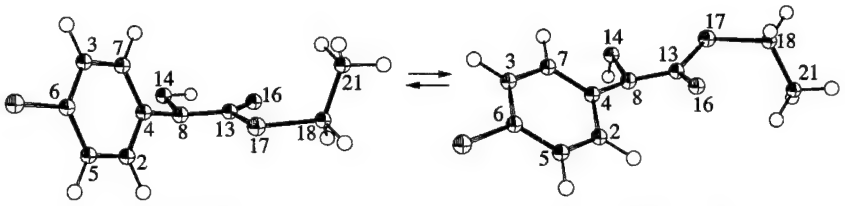


Figure 3. Geometry-optimized structures of the syn- and anti-conformations of *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)-thioethane. Critical dihedral angles are given in Table 3.

Table 3. Critical Dihedral Angles (deg) for the Geometry-Optimized Syn- and Anti-Conformers of *S*-(*N*-4-Chlorophenyl-*N*-hydroxycarbamoyl)thioethane (Figure 3) Obtained at the HF/6-31G*Level of Theory

bonds	syn-conformer	anti-conformer
C(2)−C(4)−N(8)−C(13)	−90.75	−56.27
C(2)−C(4)−N(8)−O(14)	122.76	156.48
C(7)−C(4)−N(8)−C(13)	90.78	125.49
C(7)−C(4)−N(8)−O(14)	−55.71	−21.77
C(4)−N(8)−C(13)−O(16)	−160.16	21.18
C(4)−N(8)−C(13)−S(17)	23.01	−161.26
O(14)−N(8)−C(13)−O(16)	−14.29	168.32
O(14)−N(8)−C(13)−S(17)	168.89	−14.13
relative energy (kcal/mol)	0	−3.4

The energy-minimized syn- and anti-conformers of the model compound, *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)thioethane, were calculated by both semiempirical and ab initio methods, Figure 3. The conformation of the *S*-substituent of the less stable syn-conformer is nearly identical to that of the *S*-substituent of the syn-conformer of **1d** observed in the X-ray crystal structure. At the Hartree−Fock/6-31G* level of theory, the dihedral angle between the plane of phenyl ring and that of the *N*-hydroxycarbamoyl ester function is 91°, which is close to that observed in the X-ray structure, Table 3. Apparently, the geometry of the ground-state structure of the model compound in the gas phase is determined largely by steric interactions between the ortho-protons of the ring and the *N*-OH and *S*-ethyl functions. By inference, human GlxI likely binds a low-energy conformer of syn-**1d** in which the *N*-aryl function is orthogonal to the plane of the *N*-hydroxycarbamoyl ester function. *Thus, computational methods indicate that the conformation of the S-substituent of the enzyme-bound enediol analogue is a property of the ligand and not the result of steric interactions with the enzyme protein.*

Implications for Catalysis and Inhibitor Design. The inhibitor binding studies reported here indicate the presence of a large, diffuse hydrophobic binding pocket in the active site of human GlxI that plays a major role in binding the enediol analogues to the active site. Occupancy of this pocket is necessary in order to maximize polar interactions between the enzyme and bound enediol analogue. By inference, the methyl group of GSH−methylglyoxal thiohemiacetal substrate might participate in catalysis by helping to optimize interactions between active site residues and the enediol intermediate and flanking transition states. Each of these factors must be given careful consideration in the design of future mechanism-based competitive inhibitors of GlxI as potential antitumor agents.

Experimental and Computational Methods

Synthetic methods are outlined in Scheme 1. NMR spectra were taken on a GE QE-300 NMR spectrometer. Mass spectral data were obtained at the Midwest Center for Mass Spectrometry, University of Nebraska−Lincoln, and the Washington University Mass Spectrometry Resource, Washington University−Saint Louis. Elemental analyses were obtained at Atlantic Microlabs, Inc., Norcross, GA, and are within 0.4% of the calculated values unless otherwise indicated. Oximes **5c−h** were synthesized from the corresponding aldehydes by standard methods.[23] NMR and IR analyses of the oximes matched published standard spectra. All other reagents were purchased from Aldrich.

Competitive inhibition constants were calculated from the variation in the apparent K_m of GlxI with GSH−methylglyoxal−thiohemiacetal substrate in the presence of different concentrations of enediol analogue.[9] Apparent K_m values were obtained from computer fits of the initial rate data to the Michaelis−Menten equation.

The lowest-energy gas-phase conformation of *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)thioethane was calculated using the molecular modeling program Spartan (Wavefunction, Inc., Irvine, CA).

***N*-Hydroxy-*N*-heptylcarbamate 4-Chlorophenyl Ester (3h).** This compound was prepared by a modification of a published method.[23] Heptanal oxime **5h** (1.0 g, 0.008 mol) was dissolved in 10 mL chloroform in a dry, nitrogen-flushed vessel on ice. Triethylsilane (0.90 g, 0.008 mol) was added dropwise, followed by dropwise addition of 4-chlorophenyl chloroformate (1.47 g, 0.008 mol). The mixture was stirred overnight under a nitrogen atmosphere at room temperature. The crude mixture was purified by flash gel chromatography, eluting with chloroform−methanol (40:1 to 30:1). The solvent was removed in vacuo to give a colorless oil, which crystallized to white needles on standing. The product was recrystallized from ether−hexane. Yield: 40%. Mp: 54 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.86−0.90 (m, 3H), 1.27−1.32 (m, 8H), 1.67−1.72 (m, 2H), 1.78 (s, OH), 3.65 (t, J = 7.2 Hz, 2H), 7.06 (d, J = 8.7 Hz, aromatic-2H), 7.33 (d, J = 8.7 Hz, aromatic-2H). IR (KBr): 3250, 2920, 2840, 1670, 1480, 1420, 1220, 1160, 1080, 1010, 870, 810, 750 cm^{-1}. Anal. ($C_{14}H_{20}NClO_3$) C, H, N.

***N*-Hydroxy-*N*-hexylcarbamate 4-Chlorophenyl Ester (3g).** This compound was prepared by the same general method used to prepare **3h**. The product was recrystallized from ether−hexane as colorless plates. Yield: 28%. Mp: 61−62 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.86−0.91 (m, 3H), 1.2−1.4 (m, 8H), 1.6−1.8 (m, 2H), 3.66 (t, J = 7.2 Hz, 2H), 7.06 (d, J = 8.7 Hz, aromatic-2H), 7.33 (d, J = 8.7 Hz, aromatic-2H). IR (KBr): 3250, 2900, 2840, 1660, 1480, 1420, 1220, 1160, 1080,1010,870, 810, 750 cm^{-1}. Anal. ($C_{13}H_{18}NClO_3$) C, H; N: calcd, 5.15; found, 5.08.

***N*-Hydroxy-*N*-pentylcarbamate 4-Chlorophenyl Ester (3f).** This compound was prepared by the same general method used to prepare **3h**. The product was recrystallized from ether−hexane as white rhombahedral crystals. Yield: 46%. Mp: 47−48 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.91 (t, J = 6.6 Hz, 3H), 1.28−1.37 (m, 4H), 1.72 (m, 2H), 3.67 (t, J = 6.9 Hz, 2H), 6.66 (bs, OH), 7.07 (d, J = 8.7 Hz, aromatic-2H), 7.04 (d, J = 8.7 Hz, aromatic-2H). IR (KBr): 3250, 2920, 2845, 1660, 1480, 1420, 1210, 1160, 1080, 1005,865, 810, 745 cm^{-1}. Anal. ($C_{12}H_{16}NClO_3$) C, H; N: calcd, 5.43; found, 5.37.

***N*-Hydroxy-*N*-butylcarbamate 4-Chlorophenyl Ester (3e).** This compound was prepared by the same general method used to prepare **3h**, using chloroform–ethyl acetate (100:1) as a chromatographic solvent. The product was recrystallized from ether–hexane as white flat needles. Yield: 23%. Mp: 42–43 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.93 (t, J = 7.2 Hz, 3H), 1.37 (m, 2H), 1.68 (m, 2H), 3.66 (t, J = 6.9 Hz, 2H), 7.2 (bs, OH), 7.06 (d, J = 8.7 Hz, aromatic-2H), 7.333 (d, J = 8.7 Hz, aromatic-2H). IR (KBr): 3250, 2920, 2850, 1640, 1460, 1400, 1260, 1190, 1130, 1050, 1020, 980, 845, 790, 725 cm^{-1}. Anal. ($C_{11}H_{14}NClO_3$) C; H: calcd, 5.79; found, 5.73; N: calcd, 5.75; found, 5.83.

***N*-Hydroxy-*N*-propylcarbamate 4-Chlorophenyl Ester (3d).** This compound was prepared by the same general method used to prepare **3h**, using chloroform–ethyl acetate (100:1) as a chromatographic solvent. The product was crystallized from ether–hexane as colorless flat needles. Yield: 21%. Mp: 58–59 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.97 (t, J = 7.0 Hz, 3H), 1.76 (m, 2H), 3.64 (t, J = 7.0 Hz, 2H), 6.84 (bs, OH), 7.07 (d, J = 8.8 Hz, aromatic-2H), 7.33 (d, J = 8.8 Hz, aromatic-2H). IR (KBr): 3230, 2940, 2920, 2860, 1655, 1480, 1460, 1420, 1285, 1240, 1220, 1170, 1080, 1040,1005, 865, 810, 745 cm^{-1}. Anal. ($C_{10}H_{12}NClO_3$) C; H: calcd, 5.27; found, 5.32; N: calcd, 6.10; found, 6.05.

***N*-Hydroxy-*N*-ethylcarbamate 4-Chlorophenyl Ester (3c).** This compound was prepared by the same general method used to prepare **3h**, using chloroform–ethyl acetate (100:1) as a chromatographic solvent. The product was crystallized from ether–hexane as white needles. Yield: 16%. Mp: 67–68 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 1.31 (t, J = 7.0 Hz, 3H), 3.73 (t, J = 7.0 Hz, 2H), 6.40 (bs, OH), 7.08 (d, J = 8.8 Hz, aromatic-2H), 7.34 (d, J = 8.8 Hz, aromatic-2H). IR (KBr): 3250, 2970, 2910, 2880, 2860, 1665, 1480, 1425, 1270, 1220, 1170, 1080, 1030, 1005, 960, 865, 810, 745 cm^{-1}. Anal. ($C_9H_{10}NClO_3$) C; H: calcd, 4.67; found, 4.79; N: calcd, 6.50; found, 6.38.

***N*-Hydroxycarbamate 4-Chlorophenyl Ester (3a).** This compound was prepared by reacting hydroxylamine with 4-chlorophenyl chloroformate. The product was crystallized from ether–hexane as colorless plates. Yield: 34%. Mp: 137–139 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 5.83 (bs, 1H), 7.11 (d, J = 8.7 Hz, 2H), 7.38 (d, J = 8.7 Hz, 2 H), 7.47 (bs, 1H). IR (KBr): 3300, 1725, 1465, 1260, 1210, 1080, 1005, 840 cm^{-1}. Anal. ($C_7H_6NClO_3$) C; H: calcd, 3.22; found, 3.34; N: calcd, 7.47; found, 7.39.

***S*-(*N*-Heptyl-*N*-hydroxycarbamoyl)glutathione (2h).** Into a stirring solution of 6.3 mL degassed, nitrogen-saturated ethanol:water (2:1) and **3h** (53 mg, 0.185 mmol) was placed a 6.3-fold excess of glutathione (563 mg, 1.16 mmol). The slurry was slowly brought to pH 9 by the dropwise addition of 6 N NaOH, during which the mixture became a homogeneous solution. The solution was placed under nitrogen and allowed to stand at room temperature for 24 h. The solution was brought to pH 3.5 with 6 N HCl and the solvent removed in vacuo. The white residue was suspended in 1 mL water, stirred for 9 h at room temperature, and the precipitate collected by centrifgation. This digestion procedure was repeated 4 more times. The dried white solid was then triturated 3 times with 0.8 mL portions of diethyl ether to remove unreacted **3h** and unreacted 4-chlorophenol. Yield: 35%. 300 MHz ^{1}H NMR (D_2O, pD 10.4, HOD reference): δ 0.88 (methyl-3H), 1.2–1.4 (m, alkyl-8H), 1.63 (m, alkyl-2H), 1.91 (m, Glu-C$_\beta$H$_2$), 2.40 (m, Glu-C$_\gamma$H$_2$), 3.07 (q, J = 9.0, 14.4 Hz, Cys-C$_\beta$H$_a$), 3.28 (q, J = 4.2, 14.4 Hz, Cys-C$_\beta$H$_b$), 3.32 (t, J = 6.0 Hz, Glu-C$_\alpha$H), 3.59 (t, J = 6.6 Hz, N-CH$_2$), 3.75 (d, J = 17.1 Hz, Gly-C$_\alpha$H$_a$), 3.83 (d, J = 17.1 Hz, Gly-C$_\alpha$H$_b$), 4.53 (q, J = 4.2, 9.0 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{18}H_{32}N_4SO_8$. Anal. ($C_{18}H_{32}N_4SO_8$) C, H; N: calcd, 12.06; found, 11.92.

***S*-(*N*-Hexyl-*N*-hydroxycarbamoyl)glutathione (2g).** This compound was prepared by the same general method used to prepare **2h**, using a reaction time of 70 h. Yield: 66%. 300 MHz ^{1}H NMR (D_2O, pD 10.4, DSS): δ 0.86 (methyl-3H), 1.28 (m, alkyl-6H), 1.60 (m, alkyl-2H), 1.87 (m, Glu-C$_\beta$H$_2$), 2.36 (m, Glu-C$_\gamma$H$_2$), 3.04 (q, J = 9.0, 14.4 Hz, Cys-C$_\beta$H$_b$), 3.24 (q, J = 4.2, 14.4 Hz, Cys-C$_\beta$H$_a$), 3.27 (t, J = 6.6 Hz, Glu-C$_\alpha$ H), 3.57 (t, J = 6.9 Hz, N-CH$_2$), 3.72 (d, J = 17.1 Hz, Gly-C$_\alpha$H$_a$), 3.80 (d, J = 17.1 Hz, Gly-C$_\alpha$H$_b$), 4.50 (q, J = 4.2, 9.0 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{17}H_{30}N_4SO_8$. Anal. ($C_{17}H_{30}N_4SO_8$) C, H; N: calcd, 12.44; found, 12.28.

***S*-(*N*-Pentyl-*N*-hydroxycarbamoyl)glutathione (2f).** This compound was prepared by the same general method used to prepare **2h**. Yield: 53%. 300 MHz ^{1}H NMR (D_2O, pD 10.4, DSS): δ 0.87 (methyl-3H), 1.28 (m, alkyl-4H), 1.61 (m, alkyl-2H), 1.91 (m, Glu-C$_\beta$H$_2$), 2.39 (m, Glu-C$_\gamma$H$_2$), 3.04 (q, J = 9.0, 14.4 Hz, Cys-C$_\beta$H$_b$), 3.26 (q, J = 4.2, 14.4 Hz, Cys-C$_\beta$H$_a$), 3.35 (t, J = 6.3 Hz, Glu-C$_\alpha$H), 3.57 (t, J = 6.9 Hz, N-CH$_2$), 3.73 (d, J = 17.1 Hz, Gly-C$_\alpha$H$_a$), 3.80 (d, J = 17.1 Hz, Gly-C$_\alpha$H$_b$), 4.52 (q, J = 4.2, 9.0 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{16}H_{28}N_4SO_8$. Anal. ($C_{16}H_{28}N_4SO_8$) C; H: calcd, 6.47; found, 6.38; N: calcd, 12.84; found, 12.71.

***S*-(*N*-Butyl-*N*-hydroxycarbamoyl)glutathione (2e).** This compound was prepared by the same general method used to prepare **2h**, using a reaction time of 115 h. The crude, acidified product was purified by flash chromatography on a silica gel column, using *n*-propanol:acetic acid:water (10:1:5) as an eluting solvent. The fractions containing the crude product were pooled, brought to dryness and further purified by reverse-phase column chromatography (Whatman μBondapak C_{18}, 0.78 × 30 cm), using 0.25% acetic acid and 35% methanol in water as an eluting solvent (retention volume: ~26 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 35%. 300 MHz ^{1}H NMR (D_2O, pD 3.3, DSS): δ 0.89 (t, J = 7.3 Hz methyl-3H), 1.28 (m, alkyl-2H), 1.59 (m, alkyl-2H), 2.15 (m, Glu-C$_\beta$H$_2$), 2.51 (m, Glu-C$_\gamma$H$_2$), 3.14 (q, J = 8.4, 14.3 Hz, Cys-C$_\beta$H$_b$), 3.37 (q, J = 4.8, 14.3 Hz, Cys-C$_\beta$H$_a$), 3.65 (t, J = 7.0 Hz, N-CH$_2$), 3.79 (t, J = 6.6 Hz, Glu-C$_\alpha$H), 3.94 (s, Gly-C$_\alpha$H$_2$), 4.62 (q, J = 4.8, 8.4 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{15}H_{26}N_4SO_8$. Anal. ($C_{15}H_{26}N_4SO_8 \cdot H_2O$) C: calcd, 37.86; found, 37.46; H: calcd, 5.87; found, 5.76; N: calcd, 13.58; found, 13.26.

***S*-(*N*-Propyl-*N*-hydroxycarbamoyl)glutathione (2d).** This compound was prepared by the same general method used to prepare **2e**. The flash chromatography column was eluted with *n*-propanol:acetic acid:water (70:25:30). The reverse-phase column was eluted with 0.25% acetic acid and 25% methanol in water (retention volume: ~23 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 35%. 300 MHz ^{1}H NMR (D_2O, pD 3.3, DSS): δ 0.86 (t, J = 7.3 Hz, methyl-3H), 1.63 (m, J = 7.0, J = 7.3 alkyl-2H), 2.14 (m, Glu-C$_\beta$H$_2$), 2.51 (m, Glu-C$_\gamma$H$_2$), 3.12 (q, J = 8.4, 14.3 Hz, Cys-C$_\beta$H$_b$), 3.38 (q, J = 4.8, 14.3 Hz, Cys-C$_\beta$H$_a$), 3.61 (t, J = 7.0 Hz, N-CH$_2$), 3.79 (t, J = 6.2 Hz, Glu-C$_\alpha$H), 3.94 (s, Gly-C$_\alpha$H$_2$), 4.62 (q, J = 4.8, 8.4 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{14}H_{24}N_4SO_8$. Anal. ($C_{14}H_{24}N_4SO_8 \cdot H_2O$) C; H: calcd, 6.15; found, 5.90; N: calcd, 6.50; found, 6.38.

***S*-(*N*-Ethyl-*N*-hydroxycarbamoyl)glutathione (2c).** This compound was prepared by the same general method used to prepare **2e**. The flash chromatography column was eluted with *n*-propanol:acetic acid:water (130:1:70). The reverse-phase column was eluted with 0.25% acetic acid and 5% methanol in water (retention volume: ~24 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 48%. 300 MHz ^{1}H NMR (D_2O, pD 3.3, DSS): δ 1.16 (t, J = 7.0 Hz, methyl-3H), 2.15 (m, Glu-C$_\beta$H$_2$), 2.51 (m, Glu-C$_\gamma$H$_2$), 3.12 (q, J = 8.4, 14.3 Hz, Cys-C$_\beta$H$_b$), 3.38 (q, J = 4.8, 14.3 Hz, Cys-C$_\beta$H$_a$), 3.66 (t, J = 7.0 Hz, N-CH$_2$), 3.79 (t, J = 6.2 Hz, Glu-C$_\alpha$H), 3.94 (s, Gly-C$_\alpha$H$_2$), 4.63 (q, J = 4.8, 8.4 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{13}H_{22}N_4SO_8$. Anal. ($C_{13}H_{22}N_4SO_8 \cdot H_2O$) C: calcd, 37.86; found, 37.46; H: calcd, 5.87; found, 5.76; N: calcd, 13.58; found, 13.26.

***S*-(*N*-Hydroxycarbamoyl)glutathione (2a).** This compound was prepared by the same general method used to prepare **2h**, using a 3.5-fold excess of GSH and a reaction time of 4 h. The crude reaction mixture was treated with 4-pyridine disulfide in order derivatize any unreacted GSH to the mixed

disulfide. This allowed a clean separation of the desired product by reverse-phase column chromatography using 0.25% acetic acid in water (retention volume: ~16 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 30%. 300 MHz ^{1}H NMR (D_2O, pD 3.2, DSS): δ 2.15 (m, Glu-C$_\beta$H$_2$), 2.51 (m, Glu-C$_\gamma$H$_2$), 3.20 (q, J = 8.1, 14.3 Hz, Cys-C$_\beta$H$_b$), 3.43 (q, J = 4.8, 14.3 Hz, Cys-C$_\beta$H$_a$), 3.81 (t, J = 6.2 Hz, Glu-C$_\alpha$H), 3.95 (s, Gly-C$_\alpha$H$_2$), 4.66 (q, J = 4.8, 8.1 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{11}H_{18}N_4SO_8$. Anal. ($C_{11}H_{18}N_4SO_8 \cdot H_2O$) C; H: calcd, 5.24; found, 5.17; N: calcd, 14.58; found, 14.36.

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References

(1) Thornalley, P. J. Glutathione-Dependent Detoxification of Alpha-Oxoaldehydes by the Glyoxalase System: Involvement in Disease Mechanisms and Antiproliferative Activity of Glyoxalase I Inhibitors. *Chem. Biol. Interact.* **1998**, *111−112*, 137−151.

(2) Creighton, D. J.; Hamilton, D. S.; Kavarana, M. J.; Sharkey, E. M.; Eiseman, J, L. Glyoxalase Enzyme System as a Potential Target for Antitumor Drug Development. *Drugs Future* **2000**, *25*, 385−392.

(3) Vander Jagt, D. L. The Glyoxalase System. In *Coenzymes and Cofactors: Glutathione;* Dolphin, D., Poulson, R., Avramovic, O., Eds.; John Wiley and Sons: New York, 1989; Vol. 3 (Part A), pp 597−641.

(4) Creighton, D. J.; Pourmotabbed, T. Glutathione-Dependent Aldehyde Oxidation Reactions. In *Molecular Structure and Energetics: Principles of Enzyme Activity;* Liebman, J. F., Greenberg, A., Eds.; VCH Publishers: New York, 1988; Vol. 9, pp 353−386.

(5) Richard, J. P. Kinetic Parameters for the Elimination Reaction Catalyzed by Triosephosphate Isomerase and an Estimation of the Reaction's Physiological Significance. *Biochemistry* **1991**, *30*, 4581−4585.

(6) White, J. S.; Rees, K. R. Inhibitory Effects of Methylglyoxal on DNA, RNA and Protein Synthesis in Cultured Guinea Pig Karatocytes. *Chem. Biol. Interact.* **1982**, *38*, 339−347.

(7) Papoulis, A.; Al-Abed, Y.; Bucala, R. Identification of N^2-(1-Carboxyethyl)guanine (CEG) as a Guanine Advanced Glycation End Product. *Biochemistry* **1995**, *34*, 648−655.

(8) Hamilton, D. S.; Creighton, D. J. Inhibition of Glyoxalase I by the Enediol Mimic *S*-(*N*-Hydroxy-*N*-methylcarbamoyl)glutathione: the Possible Basis of a Tumor-Selective Anticancer Strategy. *J. Biol. Chem.* **1992**, *267*, 24933−24936.

(9) Murthy, N. S. R. K.,; Bakeris, T.; Kavarana, M. J.; Hamilton, D. S.; Lan, Y.; Creighton, D. J. *S*-(*N*-Aryl-*N*-hydroxycarbamoyl)-glutathione Derivatives are Tight-Binding Inhibitors of Glyoxalase I and Slow Substrates for Glyoxalase II. *J. Med. Chem.* **1994**, *37*, 2161−2166.

(10) Kavarana, M. J.; Kovaleva, E. G.; Creighton, D. J.; Wollman, M. B.; Eiseman, J. L. Mechanism-Based Competitive Inhibitors of Glyoxalase I: Intracellular Delivery, In Vitro Antitumor Activities, and Stabilities in Human Serum and Mouse Serum. *J. Med. Chem.* **1999**, *42*, 221−228.

(11) Sharkey, E. M.; O'Neill, H. B.; Kavarana, M. J.; Wang, H.; Creighton, D. J.; Sentz, D. L.; Eiseman, J. L. Pharmacokinetics and Antitumor Properties in Tumor-bearing Mice of an Enediol Analogue Inhibitor of Glyoxalase I. *Cancer Chemother. Pharmacol.* **2000**, *46*, 156−166.

(12) Cameron, A. D.; Ridderstrom, M.; Olin, B.; Kavarana, M. J.; Creighton, D. J.; Mannervik, B. Reaction Mechanism of Glyoxalase I Explored by an X-ray Crystallographic Analysis of the Human Enzyme in Complex with a Transition State Analogue. *Biochemistry* **1999**, *38*, 13480−13490.

(13) Wu. P.-L.; Sun, C.-J. A Simple Method for the Preparation of *N*-Substituted Hydroxamic Acids by Reductive Acylation of Oximes. *Tetrahedron Lett.* **1991**, *32*, 4137−4138.

(14) Rabenstein, D. L.; Keire, D. A. The Glyoxalase System. In *Coenzymes and Cofactors: Glutathione;* Dolphin, D., Poulson, R., Avramovic, O., Eds.; John Wiley and Sons: New York, 1989; Vol. 3 (Part A), pp 67−101.

(15) Aronsson, A.-C.; Tibbelin, G.; Mannervik, B. Purification of Glyoxalase I from Human Erythrocytes by the Use of Affinity Chromatography and Separation of Three Isozymes. *Anal. Biochem.* **1979**, *92*, 390−393.

(16) Lu, T.; Creighton, D. J.; Antoine, M.; Fenselau, C.; Lovett, P. S. The Gene Encoding Glyoxalase I from *Pseudomonas putida*: Cloning, Overexpression, and Sequence Comparisons with Human Glyoxalase I. *Gene* **1994**, *150*, 93−96.

(17) Rhee, H.-I.; Murata, K.; Kimura, A. Purification and Characterization of Glyoxalase I from *Pseudomonas putida*. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 993−999.

(18) Vince, R.; Daluge, S.; Wadd, W. Studies on the Inhibition of Glyoxlase I by *S*-Substituted Glutathiones *J. Med. Chem.* **1971**, *14*, 402−405.

(19) Electronic effects appear to have little influence on binding affinity. The greater electron-withdrawing ability of the para-substituted *N*-aryl substituents, relative to the *N*-methyl function, would be expected to decrease the stability of the direct coordination interaction between the active site Zn^{2+} and the *N*-hydroxycarbamoyl ester function of the bound ligands. If this were a major factor in binding affinity, the *N*-aryl enediol analogues should bind less tightly to the enzyme than the *N*-methyl enediol analogue, contrary to observation. Electronic effects on binding of the *N*-aryl enediol analogues to the enzyme are probably small because the X-ray structure of the binary GlxI·**1d** complex shows that the plane of the *N*-aryl function is orthogonal to the plane of the *N*-hydroxycarbamoyl function. This would effectively eliminate resonance effects on the distribution of charge in the *N*-hydroxycarbamoyl ester function.

(20) Hansch, C.; Kim, K. W.; Sarma, R. H. Structure-Activity Relationship in Benzamides Inhibiting Alcohol Dehydrogenase. *J. Am. Chem. Soc.* **1973**, *95*, 6447−6449.

(21) Cameron, A. D.; Olin, B.; Ridderstrom, M.; Mannervik, B.; Jones, A. Crystal Structure of Human Glyoxalase I − Evidence for Gene Duplication and 3D Domain Swapping. *EMBO* **1997**, *16*, 3386−3395.

(22) Page, I. M.; Jencks, W. P. Entropic Contributions to Rate Accelerations in Enzymic and Intramolecular Reactions and the Chelate Effect. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678−1683.

(23) Sandler, S. R.; Karo, W. *Organic Functional Group Preparations*; Academic Press: New York, 1972; Vol. III, Chapter 11.

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Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor

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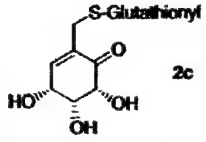
ABSTRACT



2c

The structure of the active glyoxalase I inhibitor derived from the *Streptomyces griseosporeus* metabolite COTC 1 has been conclusively identified by means of total synthesis as 2c. Human glyoxalase I is competitively inhibited by 2c ($K_i = 183 \pm 6$ μM) but is not inhibited by 1 itself.

Cytotoxic methylglyoxal is removed from cells as its glutathione adduct by the enzymes glyoxalase I and II. In 1975, a new inhibitor of glyoxalase I having structure 1 was isolated from the culture broth of *Streptomyces griseosporeus* by Umezawa and co-workers.[1] Known as COTC, compound 1 was also reported to exhibit cytotoxic and cancerostatic activity with low toxicity.[2] The potential of COTC as an anticancer drug has attracted the attention of several synthetic research groups, and five successful total syntheses have now been reported.[3−7] Despite its prospective therapeutic significance, the mechanism of action of COTC remains incompletely understood.

Umezawa et al. noted that by itself COTC had no effect on glyoxalase I, even in the presence of the substrate, methylglyoxal.[1] However, in the presence of reduced glutathione (GSH), time-dependent inhibition of the enzyme was observed. In studies with rat Yoshida glyoxalase I, 1 exhibited an apparent IC_{50} of 8.8×10^{-4} M in phosphate buffer containing methylglyoxal (0.13 M) and GSH (0.04 M). Consequently, the biological activity of COTC was thought to involve nucleophilic addition of GSH, an hypothesis later supported by the change in UV absorbance and loss of titratable SH groups noted when 1 was exposed to GSH in the absence of methylglyoxal.[8] While the reaction product of COTC with GSH has never been isolated and characterized, 1 does react with 2-mercaptoethanol and *p*-bromothiophenol to form thioethers **2a** and **2b**, respectively. Moreover, the structure of **2b** has been confirmed by X-ray crystallography.[2] On the basis of those findings, it has

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(1) Takeuchi, T.; Chimura, H.; Hamada, M.; Umezawa, H.; Yoshka, H.; Oguchi, N.; Takahashi, Y.; Matsuda, A. *J. Antibiot.* **1975**, *28*, 737−742.
(2) Chimura, H.; Nakamura, H.; Takita, T.; Takeuchi, T.; Umezawa, M.; Kato, K.; Saito, S.; Tomisawa, T.; Iitaka, Y. *J. Antibiot.* **1975**, *28*, 743−748.
(3) Mirza, S.; Molleyres, L.-P.; Vasella, A. *Helv. Chim. Acta* **1985**, *68*, 988−996.
(4) Takayama, H.; Hayashi, K.; Koizumi, T. *Tetrahedron Lett.* **1986**, *27*, 5509−5512.
(5) Shing, T. K. M.; Tang, Y. *Tetrahedron* **1990**, *46*, 6575−6584.
(6) Tatsuta, K.; Yauda, S.; Araki, N.; Takahashi, M.; Kamiya, Y. *Tetrahedron Lett.* **1998**, *39*, 401−402.
(7) Huntley, C. F. M.; Wood, H. B.; Ganem, B. *Tetrahedron Lett.* **2000**, *41*, 2031−2034.
(8) Aghil, O.; Bibby, M. C.; Carrington, S. J.; Double, J.; Douglas, K. T.; Phillips, R. M.; Shing, T. K. M. *Anti-Cancer Drug Des.* **1992**, *7*, 67−82.

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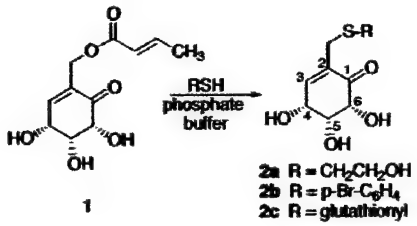
been proposed that GSH combines with **1** to form **2c**. A



1

variety of mechanisms have been suggested for this transformation, including direct displacement of crotonate,[8] two stepwise 1,4-addition/β-elimination reactions,[9] or 1,4-addition and sigmatropic rearrangement.[8] Such mechanistic speculation seemed premature in the absence of conclusive confirmation of the structure of the GSH reaction product. Here we report the isolation and purification of the GSH adduct of (−)-COTC and subsequent characterization of that structure as **2c**. In addition, we present quantitative kinetic information on the interaction of pure **2c** with glyoxalase I.

Freshly prepared synthetic (−)-**1** (13.5 mg)[7] was stirred with GSH (15.4 mg) in sodium phosphate buffer (pH 7.5) for 10 min at 37 °C and then poured onto an ion-exchange column (2 cm × 2 cm Dowex-1 resin)[10] and eluted with aqueous acetic acid to afford **2c** in 93% yield.[11] Besides the loss of crotonate and gain of glutathionyl resonances, the NMR spectrum of **2c** featured a singlet at δ 6.74 for H3 that is characteristic of β-unsubstituted cyclohexenones such as **1** and its congeners.

Adduct **2c** was a moderately potent competitive inhibitor of human erythrocyte glyoxalase I[12] (K_i = 183 ± 6 μM, Figure 1). Moreover, the inclusion of 0.2 mM COTC **1** in an assay cuvette did not reduce the initial rate of product formation beyond what would be expected from a small increase in the production of additional **2c**,[13] confirming that COTC does not inhibit the enzyme.

The fact that adduct **2c** is a moderately potent competitive inhibitor of glyoxalase I argues against an earlier suggestion

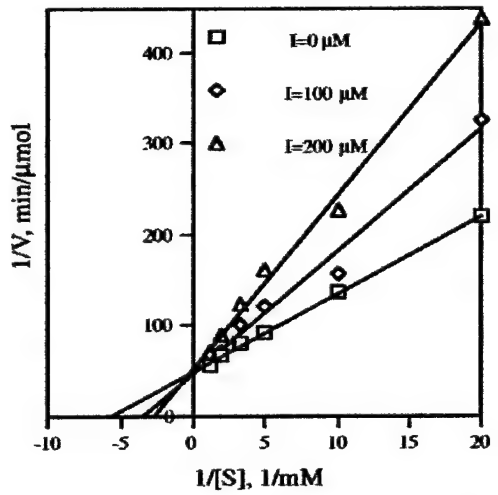


Figure 1. Reciprocal plot of the velocity of the glyoxalase I reaction (ΔA_{240}) versus the concentration of GSH-methylglyoxal thiohemiacetal ([S]) in the presence and absence of **2c** ([I]). In each kinetic run, the concentration of free GSH was maintained at 0.2 mM by varying the total concentration of GSH and methylglyoxal on the basis of the dissociation constant of the hemithioacetal (K_{diss} = 2.2 mM).[15] Conditions: 50 mM phosphate buffer, pH 7.0, 25 °C.

that COTC functions simply by depleting levels of GSH needed to form the methylglyoxal hemithioacetal substrate.[8] Studies with a variety of S-substituted glutathionyl derivatives have established that K_i values decrease with increasing hydrophobicity of the sulfur substituent. These findings suggest the presence of a hydrophobic binding pocket in the active site of human glyoxalase I, which has recently been confirmed using high-resolution X-ray crystallographic analysis.[14] The consequent possibility that less polar analogues of **2c** may bind more tightly to the enzyme will be explored further in ongoing structure−activity studies in our laboratories.

Acknowledgment. This work was supported in part by grants from the NIH (GM 24054, to B.G.; CA 59612, to D.J.C.) and the U.S. Army Medical Research and Materiel Command (to D.J.C.). Support of the Cornell NMR Facility has been provided by NSF and NIH.

OL006341Z

(9) Vasella, A.; Bandin, G.; Panza, L. *Heteroatom Chem.* **1991**, *2*, 151−161.

(10) Furano, A. V. *Methods Enzymol.* **1971**, *17B*, 509−510.

(11) For **2c**: mp 210−220 °C dec; R_f 0.40 (10:1:5 *n*-PrOH:AcOH:H_2O); ^{1}H NMR (300 MHz, D_2O) δ 6.74 (s, 1 H), 4.51 (dd, 1 H, J = 4.8, 7.0 Hz), 4.43 (m, 2 H), 3.84 (s, 2 H), 3.75 (dd, 1 H, J = 6.4, 5.9 Hz), 3.41, 3.28 (AB q, 2 H, J = 14.0 Hz), 2.95, 2.76 (ABX, 2 H, J_{AB} = 14.0, J_{AX} = 9.1, J_{BX} = 4.8 Hz), 2.48 (m, 2 H, J = 3.8, 3.9, 2.7 Hz), 2.11 (dd, 2 H, J = 7.5, 6.5 Hz); ^{13}C NMR (300 MHz, D_2O) δ 196.3, 172.2, 169.6, 144.3, 130.6, 73.4, 72.5, 64.9, 51.4, 50.2, 39.6, 29.8, 28.5, 26.3, 23.3; MS ES *m/z* 464 (M + 1, 57%), 60 (100%).

(12) Aronsson, A.-C.; Tibbelin, G.; Mannervik, B. *Anal. Biochem.* **1979**, *92*, 390−393.

(13) The rise in [**2c**] in the assay cuvette was estimated from the second-order rate constant for formation of **2c** from GSH and **1** (k = 0.12 mM^{-1} min^{-1}, pH 7, 25 °C).

(14) Cameron, A. D.; Ridderstrom, M.; Olin, B.; Kavarana, M. J.; Creighton, D. J.; Mannervik, B. *Biochemistry* **1999**, *38*, 13480−13490.

(15) Hamilton, D. S.; Creighton, D. J. *J. Biol. Chem.* **1992**, *267*, 24933−24936.

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Glutathionyl Transferase Catalyzed Addition of Glutathione to COMC: A New Hypothesis for Antitumor Activity

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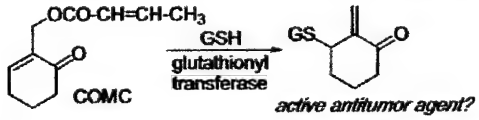
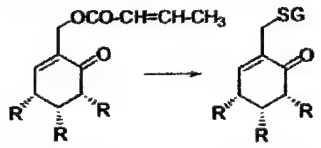
ABSTRACT



Data are presented indicating that the potent antitumor activity of 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC) and 2-crotonyloxymethyl-2-cyclohexenone (COMC) is not likely the result of glyoxalase I inhibition, as has long been assumed. An alternative hypothesis is presented, based on the finding that COMC is a substrate for human glutathionyl transferase, which produces a transient, highly electrophilic glutathionylated 2-exomethylenecyclohexanone that can covalently modify proteins and nucleic acids.

The *Streptomyces* metabolite 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC, **1a**, Scheme 1)[1,2]

Scheme 1



1a R = OH
1b R = H

2a R = OH
2b R = H

and its simpler synthetic analogue 2-crotonyloxymethyl-2-cyclohexenone (COMC, **1b**)[3] both exhibit potent antitumor activity against murine and human tumors in culture. Compound **1a** is somewhat more active ($IC_{50} = 0.5-19\ \mu M$) than **1b** ($IC_{50} = 3-44\ \mu M$).[3] In 1975, Takeuchi et al. attributed the antitumor activity of **1a** to its putative glutathione (GSH) adduct **2a**, which was proposed to inhibit the enzyme glyoxalase I (GlxI).[1]

Glyoxalase I plays a pivotal role in detoxifying intracellular methylglyoxal, which is formed during normal carbohydrate metabolism.[4] Indeed, certain inhibitors of human GlxI, the most potent of which are enediol transition state analogue inhibitors, have been shown to retard the growth of both murine and human tumors in culture and in tumor-bearing mice by causing the accumulation of intracellular methylglyoxal.[5,6]

While a priori quite plausible, the "prodrug" hypothesis of Takeuchi et al. for the antitumor action of COTC and

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(1) Takeuchi, T.; Chimura, H.; Hamada, M.; Umezawa, H.; Yoshka, H.; Oguchi, N.; Takahashi, Y.; Matsuda, A. *J. Antibiot.* **1975**, *28*, 737−742.
(2) Chimura, H.; Nakamura, H.; Takita, T.; Takeuchi, T.; Umezawa, M.; Kato, K.; Saito, S.; Tomisawa, T.; Iitaka, Y. *J. Antibiot.* **1975**, *28*, 743−748.
(3) Aghil, O.; Bibby, M. C.; Carrington, S. J.; Double, J.; Douglas, K. T.; Phillips, R. M.; Shing, T. K. M. *Anti-Cancer Drug Des.* **1992**, *7*, 67−82.
(4) Creighton, D. J.; Pourmotabbed, T. In *Molecular Structure and Energetics: Principles of Enzyme Activity*; Liebman, J. F., Greenberg, A., Eds.; VCH Publishers: New York 1988; Vol. 9, pp 353−386.
(5) Kavarana, M. J.; Kovaleva, E. G.; Creighton, D. J.; Wollman, M. B.; Eiseman, J. L. *J. Med. Chem.* **1999**, *42*, 221−228.
(6) Sharkey, E. M.; O'Neill, H. B.; Kavarana, M. J.; Wang, H.; Creighton, D. J.; Sentz, D. L.; Eiseman, J. L. *Cancer Chemother. Pharmacol.* **2000**, *46*, 156−166.

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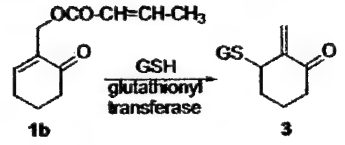
COMC has never been tested. Recently, an authentic sample of **2a** was prepared by independent synthesis and shown to be a modest inhibitor of GlxI.[7]

We have now prepared and tested **2b** against human erythrocyte GlxI and report that, like **2a**, it is several orders of magnitude weaker than other, mechanism-based, GlxI inhibitors that display potent antitumor activity.[5,6] To establish an alternative mode of tumor toxicity for COTC and COMC, we have developed a short and efficient synthesis of **1b** and have now demonstrated that it is a substrate for glutathionyl transferase, an enzyme that is widely distributed in mammalian tissue.

Here we show that glutathionyl transferase catalyzes a conjugate addition of GSH to **1b** with concomitant elimination of crotonic acid leading to 3-glutathionyl-2-exomethylenecyclohexanone **3** (Scheme 2), a reactive intermediate that

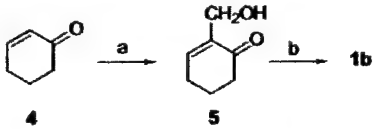
Scheme 2



has now been isolated and characterized for the first time. Trapping studies with amino acids and nucleotides support the hypothesis that **3** can react with and covalently modify reactive groups on proteins and DNA that may be critical to cell viability.

Scheme 3 depicts a simple and efficient synthesis of **1b** that is superior to the earlier reported seven-step procedure.[3] Baylis–Hillman reaction of 2-cyclohexenone **4** with formaldehyde afforded 2-hydroxymethyl-2-cyclohexenone **5**,[8] which was then crotonylated to **1b** following the literature procedure.[3]

Scheme 3



(a) DMAP, CH_2O, THF, rt 65%; (b) crotonic anhydride, pyridine, DMAP, rt 92%.

The GSH conjugate **2b** was prepared from **1b** by adapting the procedure for making **2a** from **1a**.[7] The NMR spectrum of **2b** featured the expected glutathionyl resonances and the downfield resonance (δ 7.12, triplet) characteristic of H3 in β,γ-unsubstituted 2-cyclohexenones.

Kinetic studies with human erythrocyte GlxI (sodium phosphate buffer, pH 7.0, 25 °C) indicated that **2b** was a competitive inhibitor of the enzyme, with a dissociation constant $K_i = 107 \pm 1$ μM. We have previously reported that **2a** competitively inhibited human erythrocyte GlxI, with a dissociation constant $K_i = 183 \pm 6$ μM.[7]

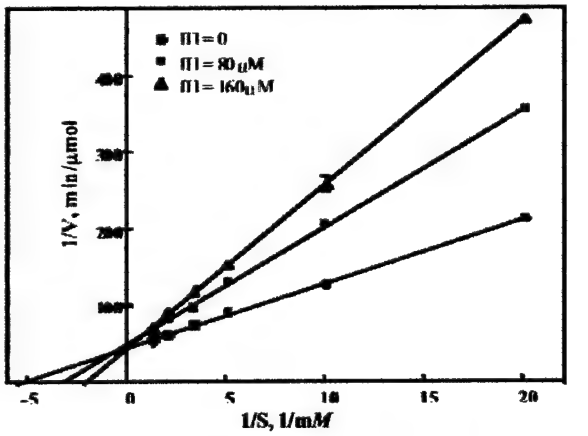


Figure 1. Reciprocal plot of the velocity of the glyoxalase I reaction (ΔOD_{240}) versus the concentration of GSH-methylglyoxal-thiohemiacetal (S) in the absence and presence of different concentrations of **2b**. In each kinetic run, the concentration of free GSH was maintained at 0.2 mM by varying the total concentration of GSH and methylglyoxal on the basis of the dissociation constant of the hemithioacetal (K_{diss} = 2.2 mM).[9] Conditions: 50 mM phosphate buffer, pH 7.0, 25 °C.

To investigate the mechanism of formation of **2b**, the nonenzymatic reaction of **1b** with GSH was monitored spectrophotometrically and followed a simple first-order decay (Figure 2, trace A). No intermediate species was detectable. However, in the presence of human placental glutathione transferase (GSTP1-1)[10] the reaction rate profile

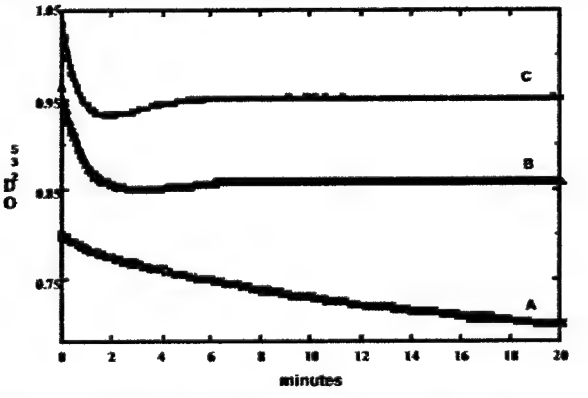
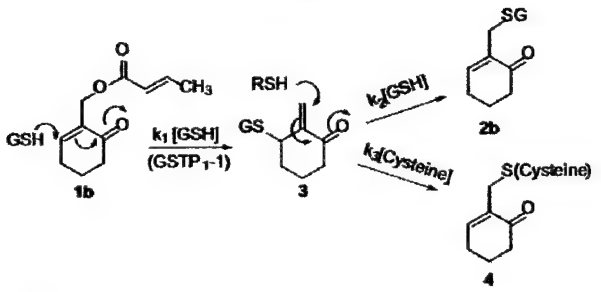


Figure 2. Spectrophotometrically determined rates of reaction of GSH (1.03 mM) with **1b** (0.05 mM) (A) in the absence of GSTP1-1 ($k = 0.070 \pm 0.0002$ min^{-1}); (B) in the presence of 1.8 units of GSTP1-1 ($k_1 = 0.882 \pm 0.055$ min^{-1}; $k_2 = 0.633 \pm 0.053$ min^{-1}); and (C) in the presence of 2.4 units of GSTP1-1 ($k_1 = 1.33 \pm 0.037$ min^{-1}; $k_2 = 0.685 \pm 0.024$ min^{-1}). Conditions: 100 mM phosphate buffer, 0.05 mM EDTA, pH 6.5, 25 °C.

conformed to a double exponential decay, composed of a rapid, enzyme-dependent, initial phase involving **1b** followed by a slower enzyme-independent first-order phase (Figure 2, traces B and C).

This finding was consistent with the mechanism shown in Scheme 4, whereby an initial, enzyme-catalyzed Michael addition of GSH to **1b** afforded the exocyclic enone **3**. Once dissociated from the enzyme, free **3** reacted with GSH nonenzymatically to form **2b**.

Scheme 4



When **1b** (0.1 mM) was incubated with cysteine (0.5 mM) and GSH (0.5 mM) in the presence of GSTP1-1 (1.5 units) for 30 min and the reaction mixture was fractionated by reverse-phase HPLC, thiol adducts **2b** and **4** were isolated. On the basis of the integrated intensities of the well-resolved peaks corresponding to **4** (~9.5 min) and **2b** (~11.5 min), the product ratio in the presence of GSTP1-1 was identical to that obtained from a nonenzymatic incubation of **1b** with the same concentrations of cysteine and GSH (Figure 3).

Figure 3. Fractionation of product mixtures in the absence and presence of GSTP1-1 by reverse-phase HPLC (Waters, μ-Bondapak C_{18}, 0.78 × 30 cm).

That finding indicated that **3** dissociated from the enzyme prior to reacting with the free thiols in solution and was consistent with the mechanism in Scheme 4. Adduct **2b** (0.5 mM) was stable under the reaction conditions, undergoing less than 1% conversion to **4** in the presence of cysteine (2.5 mM) over 27 h, as determined by HPLC.

Initial rates of reaction of **1a** and **1b** with GSTP1-1 were determined from reciprocal plots of initial velocities (ΔOD_{235}/min), versus [substrate] in buffered solution at pH 6.5, with [GSH] = 1 mM (25 °C). Under those conditions, the enzyme-catalyzed GSH addition became rate determining (<0.01 units of transferase in the assay cuvettes). For **1b**, $k_{cat} = 1.2 \pm 0.2$ s^{-1} and $K_m = 52 \pm 10$ μM. For **1a**, the individual kinetic constants could not be accurately determined, although k_{cat}/K_m was estimated to be 8.3-fold lower than that of **1b**.

Brief incubation of a mixture of **1b**, GSH, and GSTP1-1 gave rise to a transient intermediate that could be isolated by reverse-phase HPLC, with a retention time close to that of synthetic **2b**. The kinetic properties of this species suggest that it is an obligatory intermediate associated with the enzymatic conversion of **1b** to **2b**. When this species is combined with cysteine in buffered solution (pH 7), a new species is produced that comigrates with authentic **4**. Moreover, incubation of this intermediate species with excess GSH (1.04 mM) in potassium phosphate buffer (0.1 M, pH 6.5) at 25 °C results in a first order *increase* in OD_{235} with a rate constant of 0.87 ± 0.08 min^{-1} similar in magnitude to that associated with the second phase of the transferase-catalyzed conversion of **1b** to **2b**, Figure 2. The absorptivity of the intermediate ($\epsilon_{235} = 4300$ cm^{-1} M^{-1}) is significantly less than that of the final product **2b** ($\epsilon_{235} = 7500$ cm^{-1} M^{-1}), which accounts for the overall shape of the reaction-rate profile in the presence of transferase (Figure 2).

The 600-MHz ^{1}NMR spectrum of the intermediate was consistent with the structure of **3** (Figure 4). The vinyl proton resonances at 5.76 and 5.29 ppm were characteristic of

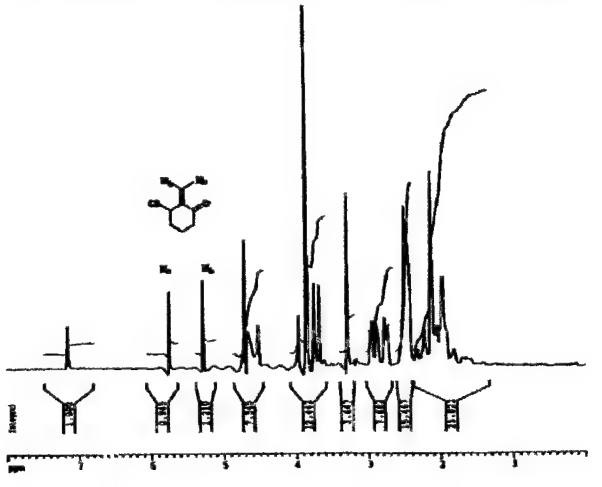


Figure 4. The 600-MHz ^{1}H NMR spectrum of intermediate **3**.

geminal vinylic hydrogens and consistent with published NMR spectra of several closely related 2-methylenecyclohexanones.[11] Other resonances in the spectrum corresponded to those expected for the tripeptide moiety.[12] The resonance at δ 7.12 indicated the presence of **2b**, which was formed in the transferase-independent addition of GSH to **3**.

Comparative data analysis suggested that the potent antitumor activities of COTC (**1a**) and COMC (**1b**) could not be rationalized by the biological activity of **2a** and **2b**, respectively, which are weak competitive inhibitors of human erythrocyte GlxI. Earlier in vitro studies on enediol analogue inhibitors of GlxI that inhibited the growth of L1210 and B16 melanotic melanoma as ester prodrugs revealed K_i values against GlxI in the submicromolar range.[6] In fact, a direct correlation was observed between the K_i values of the prodrugs against GlxI and their corresponding antitumor activities, as measured by IC_{50} values. For example, the weakest GlxI inhibitor in that study ($K_i = 0.16\ \mu M$) displayed an IC_{50} value $>100\ \mu M$. On the basis of that correlation, the potent antitumor activity of **1b** ($IC_{50} = 0.5-19\ \mu M$) is inconsistent with the hypothesis of Takeuchi et al. attributing antitumor activity to the action of **2b** as GlxI inhibitor ($K_i = 107\ \mu M$).[1]

Besides providing insight into the mechanism of addition of GSH to COMC, the formation of **3** enzymatically from **1b** also offers an attractive alternative biological mechanism for the tumoricidal activity of both COTC and COMC. It may now be hypothesized that **1a** and **1b** are enzyme-activated prodrugs in which the crotonate ester serves as a leaving group, in a process triggered by glutathionyl transferase. Methylenecyclohexanones such as **3** are known to be highly reactive Michael acceptors[11] and can function as carcinostatic agents by reacting with proteins and nucleic acids critical to cell function. Besides the thiol adducts documented here, covalent adducts between **1b** and model polynucleic acids in the presence of GSTP1-1 have been detected by mass spectrometry.[13]

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Note Added after Print Publication: Due to a production error, certain μM concentrations appeared as mM concentrations in the version published on the Web 03/03/2002 (ASAP) and in the April 4, 2002 issue (Vol. 4, No. 7, pp 1209−1212); the correct electronic version of the paper was published on 04/23/2002 and an Addition and Correction appears in the May 16, 2002 issue (Vol. 4, No. 10).

Supporting Information Available: Experimental procedures and ^1H and ^{13}C NMR data for the synthesis of **1b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Huntley, C. F.; Hamilton, D. S.; Creighton, D. J.; Ganem, B. *Org. Lett.* **2000**, *2*, 3143−3144.

(8) Rezgui, F.; El Gaied, M. M. *Tetrahedron Lett.* **1998**, *39*, 5965−5966.

(9) Hamilton, D. S.; Creighton, D. J. *J. Biol. Chem.* **1992**, *267*, 24933−24936.

(10) Predominantly the pi or P1-1 isoform, purchased from Sigma Chemical Company. Salts and free GSH were removed by ultrafiltration. Units of transferase activity were determined using 1-chloro-2,4-dinitrobenzene as substrate: Mannervik, B.; Danielson, U. H. *Crit. Rev. Biochem.* **1988**, *23*, 283−337.

(11) Tamura, R., Watabe, K., Ono, N., Yamamoto, Y. *J. Org. Chem.* **1992**, *57*, 4895−4903.

(12) Rabenstein, D. L.; Keire, D. A. In *Coenzymes and Cofactors: Glutathione*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; John Wiley: New York, 1989; Vol. 3, Part A, pp 67−101.

(13) Fabris, D.; Creighton D. J.; Ganem, B. Unpublished work.

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Alkylation of Nucleic Acids by the Antitumor Agent COMC

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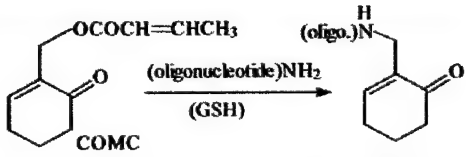
ABSTRACT



Mass spectral data are presented indicating that the antitumor agent 2-crotonyloxymethyl-2-cyclohexenone (COMC) is capable of alkylating oligonucleotides via a mechanism involving an electrophilic exocyclic enone intermediate. Under physiological conditions, the exocyclic enone is likely the glutathionylated 2-exomethylenecyclohexenone. This supports a recent hypothesis that the antitumor activity of COMC arises from alkylation of nucleic acids and/or proteins critical to cell function and not from competitive inhibition of glyoxalase I by an adduct of COMC and glutathione.

2-Crotonyloxymethyl-2-cyclohexenone (COMC, **1**) is a synthetic analogue of the *Streptomyces* metabolite 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC).[1,2] Both compounds exhibit potent antitumor activity *in vitro* and *in vivo*.[3] Early investigators proposed that antitumor activity might arise from competitive inhibition of the methylglyoxal detoxifying enzyme glyoxalase I by the covalent adducts resulting from displacement of crotonate by intracellular glutathione (GSH).[1]

The recent development of simple, high-yield synthetic routes to the GSH adducts of COMC/COTC allowed a careful quantitative assessment of their ability to inhibit human glyoxalase I.[4,5] Surprisingly, the adducts proved to be relatively poor inhibitors of human glyoxalase I, casting serious doubts on the involvement of the enzyme in the antitumor activity of COTC/COMC. Kinetic measurements and intermediate trapping experiments also indicated that the mechanism of formation of the GSH adducts involves a multistep mechanism (Scheme 1) in which COMC (**1**) undergoes an initial Michael addition by 1 equiv of GSH to give a highly reactive exocyclic enone (**3**), a process that is catalyzed by human GSH transferase (hGST).[4] The intermediate then reacts with a second equivalent of GSH to give the final product (**5**). This chemistry might also account for the cytotoxicity of COMC, as the exocyclic enone is likely to react with nucleophilic groups on nucleic acids and proteins inside cells. Indeed, DNA alkylation, in particular, provides a plausible mechanism of cytotoxic activity, which is shared by a large number of chemical carcinogens and cross-linking antitumor agents.[6,7] Here we present direct evidence in support of this chemical mechanism in the case

[†] University of Maryland, Baltimore County.
[‡] Cornell University.

(1) Takeuchi, T.; Chimura, H.; Hamada, M.; Umezawa, H.; Yoshka, H.; Oguchi, N.; Takahashi, Y.; Matsuda, A. A. *J. Antibiot.* **1975**, *28*, 737−742.
(2) Chimura, H.; Nakamura, H.; Takita, T.; Takeuchi, T.; Umezawa, M.; Kato, K.; Saito, S.; Tomisawa, T.; Iitaka, Y. *J. Antibiot.* **1975**, *28*, 743−748.
(3) Aghil, O.; Bibby, M. C.; Carrington, S. J.; Double, J.; Douglas, K. T.; Phillips, R. M.; Shing, T. K. M. *Anti-Cancer Drug Des.* **1992**, *7*, 67.
(4) Huntley, C. F.; Hamilton, D. S.; Creighton, D. J.; Ganem, B. *Org. Lett.* **2000**, *2*, 3143−3144.
(5) Hamilton, D. S.; Ding, Z.; Ganem, B.; Creighton, D. J. *Org. Lett.*, in press.
(6) Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723−2795.
(7) *Chemical Carcinogens and DNA*; Searle, C. E., Ed.; Monograph 173, American Chemical Society: Washington, DC, 1979.

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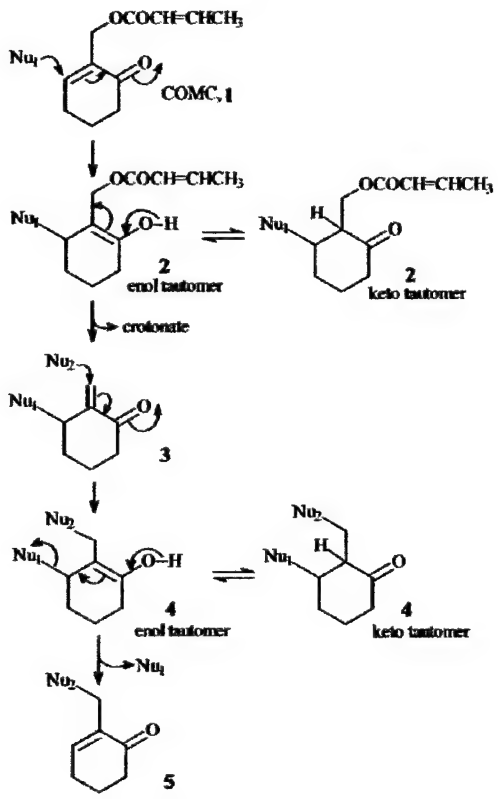
Scheme 1



of COMC. The formation *in vitro* of a reactive glutathionyl exocyclic enone intermediate and its ability to produce adducts with nucleic acids has been confirmed by direct mass spectrometric analysis of the reaction mixtures.

Direct infusion electrospray ionization with an ion cyclotron resonance (ICR) analyzer[8,9] was employed to identify the products of the *in vitro* reaction of COMC with GSH and with dinucleotides and oligonucleotides.[10] Generally, reaction mixtures were made up in 10 mM ammonium acetate buffer (pH 7.0) containing 3.3 mM COMC, 1.3 mM GSH, and 1.3 mM dinucleotide or oligonucleotide. Reaction mixtures were incubated at room temperature for approximately 1 h, quenched by the addition of one volume of methanol, and stored at −20 °C.

For a reaction mixture containing COMC and GSH, protonated ions corresponding to the species shown in Scheme 1 ($Nu_1 = Nu_2 = GSH$) were detected in positive ion mode (Figure 1). The same spectrum was obtained in the presence of 1.2 units of hGST.[11] For each signal, the experimental mass over charge (*m/z*) value was in excellent agreement with the calculated monoisotopic mass (Table 1).

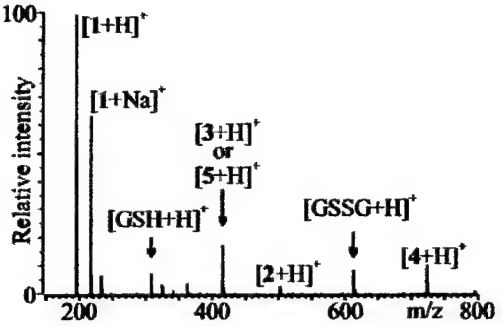


Figure 1. Positive ion mode ESI FT-MS spectrum of the product mixture initially composed of COMC and GSH in the presence of 1.2 units human glutathione-S-transferase, under the conditions given in the text.

Table 1. Summary of the Molecular Mass Determination of Reaction Products of COMC with GSH and Guanosylyl (3′−5′)Adenosine (GA)

product	elemental composition (neutral)	expected monoisotopic mass	observed monoisotopic mass[a]
COMC, **1**	$C_{11}H_{14}O_3$	195.1021[b]	195.1007[b]
GSH	$C_{10}H_{17}N_3O_6S$	308.0916[b]	308.0913[b]
species **3**,**5**	$C_{17}H_{25}N_3O_7S$	416.1491[b]	416.1486[b]
species **2**	$C_{21}H_{31}N_3O_9S$	502.1859[b]	502.1856[b]
GA	$C_{20}H_{25}N_{10}O_{11}P$	611.1364[c]	611.1140[c]
GSSG	$C_{20}H_{32}N_6O_{12}S_2$	613.1598[b]	613.1593[b]
GA+hex[d]	$C_{27}H_{33}N_{10}O_{12}P$	719.1939[c]	719.1888[c]
species **4**	$C_{27}H_{42}N_6O_{13}S_2$	723.2330[b]	723.2343[b]
GA+COMC	$C_{31}H_{39}N_{10}O_{14}P$	807.2463[b]	807.2472[b]
GA+GSH+hex[d]	$C_{37}H_{50}N_{13}O_{18}PS$	1026.278[c]	1026.240[c]
2GA+hex[d]	$C_{47}H_{58}N_{20}O_{23}P_2$	1331.338[c]	1331.258[c]

[a] The average mass accuracy was determined to be 12 ppm. [b] Protonated ($[M + H]^+$) species observed in positive ion mode. [c] Deprotonated ($[M - H]^-$) species observed in negative ion mode. [d] 2-Methylene-2-cyclohexenone.

Ions were detected for starting reagents COMC and GSH, together with oxidized glutathione (GSSG), which was also present in the initial stock solution of GSH. Adduct **2**, either as the enol- or keto-tautomer, gave a weak but recognizable signal. The 1 h reaction time is sufficient for the reaction to go nearly to completion but is probably short enough to allow for the detection of some residual keto-tautomer arising from

(8) Comisarow, M. B.; Marshall, A. G. *Chem. Phys. Lett.* **1974a**, *25*, 282−283.

(9) Marshall, A. G.; Hendrickson, C. L.; Jackson, G. S. *Mass Spectrom. Rev.* **1998**, *17*, 1−35.

(10) Mass spectrometric analyses were performed on a Bruker Daltonics (Billerica, MA) Apex III Fourier Transform mass spectrometer (FT-MS) equipped with a 7T actively shielded superconductive magnet and Apollo electrospray ionization (ESI) source. Positive ion determinations were obtained in water, methanol, glacial acetic acid (49:49:2); negative ion determinations in methanol and 10 mM ammonium acetate (1:1). Solutions were continuously infused through a syringe pump at a flow rate of 2 μL/min. Tandem experiments were performed by sustained off-resonance irradiation (SORI) of selected precursor ions. Intensity and duration of off-resonance irradiation, type and pressure of collision gas (Ar), and duration of the collisional step were kept constant.

(11) hGST was purchased from Sigma Chem. Co. and is composed primarily of the GSTP1-1 isozyme.

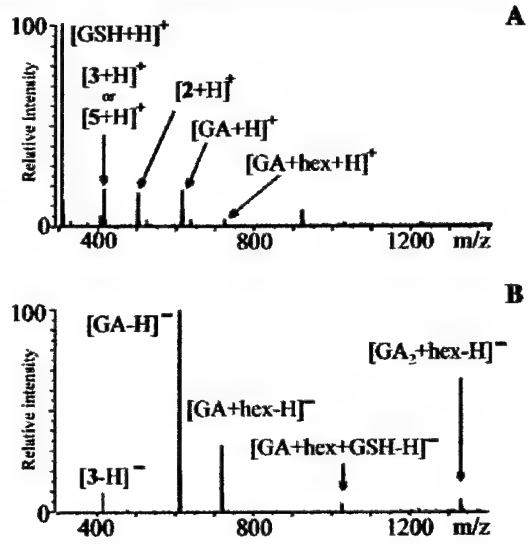


Figure 2. (A) Positive ion and (B) negative ion mode ESI FT-MS spectra of the product mixture initially composed of COMC, GA, and GSH. The abbreviation "hex" indicates the alkylating 2-methylene-2-cyclohexenone groups.

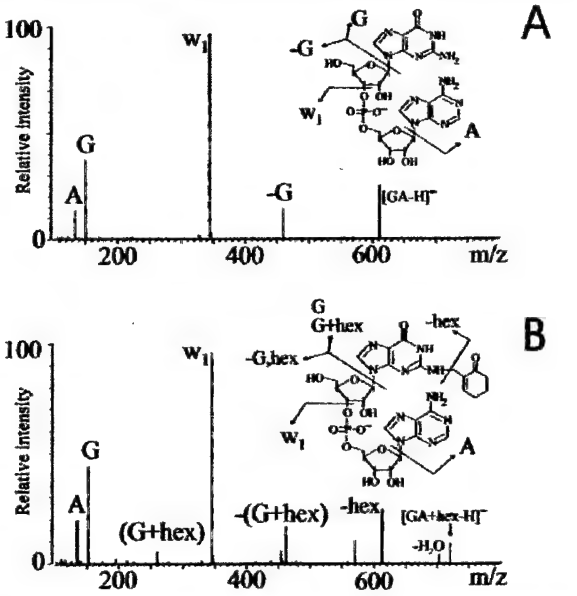


Figure 3. Collision-induced dissociation spectra of (A) deprotonated GA and (B) deprotonated COMC-GA adduct. To facilitate the interpretation, the putative structure with the proposed fragmentation pattern is included. The abbreviation "hex" indicates the 2-methylene-2-cyclohexenone group.

the unstable enol species formed along the reaction pathway. Similarly, the GSH bis-adduct **4** is most likely detected as the more stable keto-tautomer.

The isobaric (same mass) species **3** and **5**, having the same elemental composition, could not be distinguished on the basis of an accurate mass determination. In an analogous way, tandem mass spectrometry proved unsuccessful, as the ion corresponding to the **3/5** species gave a fragmentation pattern in reasonable agreement with either structural isomer. Nevertheless, the detection of ions corresponding to both **2** and **4** strongly argues for the Michael addition mechanism shown in Scheme 1. Therefore, a simple S_N2 mechanism seems inconsistent with the mass spectral data.

To assess the reactivity of 2-cyclohexenone derivatives with nucleic acids, COMC and GSH were incubated in the presence of ribo-dinucleotides[12] of different base composition. The products of the reaction with guanosylyl(3′-5′)-adenosine (GA) are shown in Figure 2 and summarized in Table 1. As expected, species **2**, **3/5**, and **4** (with Nu_1 = Nu_2 = GSH) are readily recognizable in the reaction mixture analyzed in positive ion mode (Figure 2A). However, only weak signals could be detected for protonated GA and GA-adducts. A more favorable signal-to-noise ratio was obtained in negative ion mode (Figure 2B), due to the negatively charged phosphate of the nucleotide function. The switch of polarity allowed the detection of abundant GA-COMC adducts (with Nu_1 = Nu_2 = GA) and bis-adducts, with and without GSH (**4**, $Nu_{1,2}$ = GSH and including either Nu_1 = Nu_2 = GA, or different permutations of $Nu_{2,1}$ = GA, respectively).

It is important to note that GSH is not a strict requirement for adduct formation with the dinucleotides, as the bis-adduct $[GA_2 + hex\text{-}H]^-$ is present in the spectrum shown in Figure 2B. Indeed, when the reaction is repeated in the absence of GSH, both the mono- and bis-adducts of the dinucleotides are observed (data not shown).

To identify the functional group(s) involved in the alkylation process, the dinucleotides AA, GG, CC, UU, and TT (as 2′-deoxy-ribonucleotides) were tested with COMC. All substrates produced adducts with the exception of UU and TT. Thus, the likely sites of alkylation are the exocyclic amino groups of the nitrogenous bases, which are known to be favorable targets of electrophilic attack by genotoxic carcinogens.[13]

As one of the leading techniques employed to identify nucleic acids modifications and to localize the sequence position of covalently modified bases,[14,15] tandem mass spectrometry was applied here to characterize the COMC-GA conjugate. Initially, collision-induced dissociation was performed on GA, which showed the normal fragmentation pattern provided by nucleic acids (Figure 3A). Typically, the nitrogenous base at the 5′-end is lost through facile cleavage of the N-glycosidic bond between C-1′ of the pentose and N-9 of the base.[16−18] Subsequently, the bond

(12) Di- and oligonucleotides were purchased from the Keck DNA Synthesis Facility of Yale University and used without further purification.

(13) Phillips, D. H. In *The Molecular Basis of Cancer*; Farmer, P. B., Walker, J. M., Eds.; Wiley-Interscience: London, UK, 1985; pp 133−179.

(14) Barry, J. P.; Vouros, P.; Van Schepdael, A.; Law, S.-J. *J. Mass Spectrom.* **1995**, *30*, 993−1006.

(15) Beck, J. L.; Colgrave, M. L.; Ralph, S. F.; Sheil, M. M *Mass Spectrom. Rev.* **2001**, *20*, 61−87.

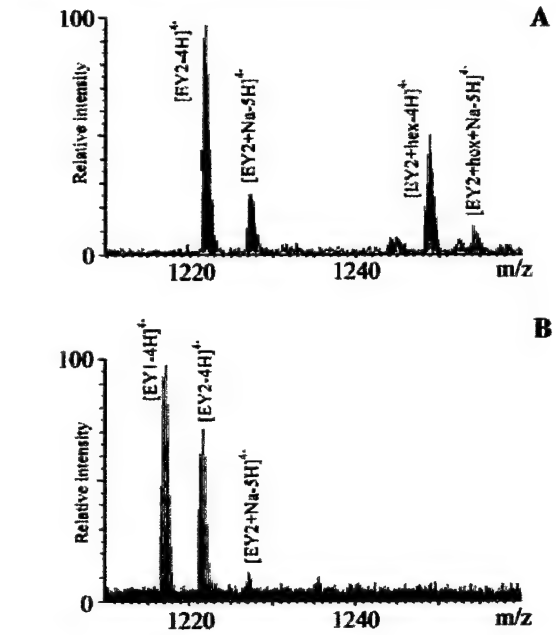


Figure 4. Negative ion mode ESI FT-MS spectra of the reaction of COMC with (A) the 16mer oligonucleotide EY2 (ACG GAC CGG CGT ACG C) and (B) a duplex formed after annealing with its complementary EY1 (GCG TAC GCC GGT CCG T). Only the region including the −4 charge state is shown for better comparison.

between C-3′ and O-3′ of the abasic pentose is cleaved, causing the scission of the phosphodiester backbone and producing the sequence ions employed for gas-phase sequencing of nucleic acids.[16−18]

In the case of GA, the initial fragmentation event resulted in the formation of the species indicated as G and its complementary ion −G (depending on which moiety retained the charge when scission was complete), and then the sequence ion w_1 was formed by loss of the abasic pentose. The loss of adenine base was also observed, although with lower abundance than the loss of guanine, but the cleavage of the C−O bond at the 3′ end was not.

The fragmentation pattern provided by the COMC-GA adduct followed the same pattern, but the mass of characteristic fragments was shifted by the addition of COMC, helping to locate the 2-methylene-2-hexenone group on the guanine base (Figure 3B). In particular, guanine base was lost as alkylated guanine and detected as (G+hex) or as the complementary ion −(G+hex). The loss of nonalkylated base G was also observed and could be explained by consecutive cleavage reactions including the loss of COMC. To the contrary, adenine was only lost as unmodified base A. Moreover, the sequence ion w_1 (encompassing the 3′-adenylyl moiety) afforded the same mass observed for COMC-free GA, thus excluding the possibility of alkylation on the 3′-nucleotide.

The specificity of COMC was also evaluated with oligonucleotides of different length and base composition, either in their single stranded form or annealed with their complementary oligonucleotide to form a duplex. For example, adduct formation was readily observed when the single stranded DNA 16mer EY2 (ACG GAC CGG CGT ACG C) was treated with COMC (Figure 4A). The molecular mass was determined to be 4889.815 Da (4889.850 Da calculated from sequence for the ^{12}C monoisotopic species) for the starting 16mer and 4997.901 Da (4997.907 Da from sequence) for the alkylated product. However, when the same reaction was carried out after annealing of EY2 with the complementary 16mer EY1 (GCG TAC GCC GGT CCG T), no alkylation was detected on either strand (Figure 4B). This finding suggests that the COMC function is attached to an exocyclic amino group, which is sterically protected by base pairing in the duplex oligonucleotide, and effectively rules out the purines C8 position as a possible reactive site for cyclohexenone addition.

In conclusion, COMC forms stable adducts *in vitro* with GSH and with nucleic acids. Adducts can form by either enzymatic or nonenzymatic pathways, following the addition−elimination mechanism shown in Scheme 1. This mechanism is strongly supported by the observation of key intermediates along the reaction pathway.

Alkylation of nucleic acids occurs both in the presence and in the absence of GSH/hGST and follows the same general reaction pathway. The site of alkylation appears to be the exocyclic amino groups of adenine, cytosine, and guanine. Future confirmation by other means of structural determination (*e.g.*, NMR) would be desirable. Conceivably, 2-hexenone derivatives may exhibit sequence specificity, on the basis of the observation that COMC preferentially modifies guanine in GA. Indeed, genotoxic alkylating agents have previously been reported to exhibit specificity toward GC-rich regions in oligonucleotides.[19] This observation suggests the possible existence of hypersensitive motifs toward alkylation by COMC and its derivatives.

Finally, the observations reported here clearly suggest that the cytotoxic activity of COMC and its derivatives may well arise from alkylation of polynucleic acids critical to cell function. Given the high concentration of GSH in cells under physiological conditions, GSH is likely to be involved in the initial activation step (Scheme 1). However, a clear assessment of GSH/GST activation *vs* direct COMC alkylation will require kinetics investigations.

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OL025612Y

(16) McLuckey, S. A.; Habibi-Goodarzi, S. *J. Am. Chem. Soc.* **1993**, *115*, 12085−12095.

(17) Little, D. P.; Chorush, R. A.; Spier, J. P.; Senko, M. W.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1994**, *116*, 4893−4897.

(18) Ni, J.; Pomerantz, C.; Rozenski, J.; Zhang, Y.; McCloskey J. A. *Anal. Chem.* **1996**, *68*, 1989−1999.

(19) Mattes, W. B.; Hartley, J. A.; Kohn, K. W.; Matheson, D. W. *Carcinogenesis* **1988**, *9*, 2065−2072.

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Abstract Number: 368

In Vitro Antitumor activity of endocyclic enone compounds: 2-crotonyloxymethyl-2-cyclohexenone (COMC-6),-cyclopentenone (COMC-5) or -cycloheptenone (COMC-7) in B16 murine melanoma and HT29 human colon adenocarcinoma

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The antitumor activity of 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) has been attributed to the intracellular conjugate of glutathione with COMC-6 (COMCG), which might inhibit the detoxifying enzyme, glyoxalase I. In order to test this hypothesis, we have determined the *in vitro* cytotoxicity of COMC-6; $COMCG(Et)_2$, an alternative prodrug which is the diethyl ester of COMCG; COMC-5; and COMC-7 against B16 murine melanoma cells. The cytotoxicity of COMC-6 was also examined in $HT29^{par}$ human colon adenocarcinoma cells and $HT29^{MDR}$ cells, which overexpress p-glycoprotein. Cytotoxicities were determined after 72 h exposure of cells in culture to 6 concentrations of each compound. Viability was determined by trypan blue exclusion and cells were counted by hemocytometer. In addition, we examined the time course of accumulation of COMCG in B16 cells after exposure to either COMC-6 or $COMCG(Et)_2$. Accumulation studies were carried out by incubating cells with 50 ?M compound for 30 sec, 10, 30, 60 or 120 min. Cells were spun through silicone oil to remove extracellular medium. Cell pellets were lysed, extracted with ethanol, and fractionated by HPLC. The IC_{50}'s of COMC-5, COMC-6, and COMC-7 in B16 murine melanoma cells were respectively: 0.14, 0.041, and 0.029 ?M. In contrast, $COMCG(Et)_2$ was much less toxic (IC_{50}, 460 ?M). The IC_{50} of COMC-6 against $HT29^{par}$ human colon adenocarcinoma cells was 0.81 ?M; while in $HT29^{MDR}$ cells the IC_{50} was 2.70 ?M, a change of only 3-fold compared to the 20-fold difference in cytotoxicity of vincristine under the same conditions. After incubation with COMC-6, there was significant intracellular accumulation of COMCG at 30 sec and 10 min, 0.041 and 0.074 nmol, respectively. However, no COMCG was detected at later time points. After incubation with $COMCG(Et)_2$, COMCG was only detected at 30 sec (0.037 nmol), but not at later time points. Although both COMC-6 and $COMCG(Et)_2$ result in intracellular COMCG, only COMC-5, COMC-6, or COMC-7 are toxic to the cells. This suggests that cytotoxicity may arise from something other than the adduct, perhaps a reactive intermediate formed during conjugation. Support: DAMD17-99-1-9275.

Molecular Basis of the Antitumor Activities of 2-Crotonyloxymethyl-2-cycloalkenones

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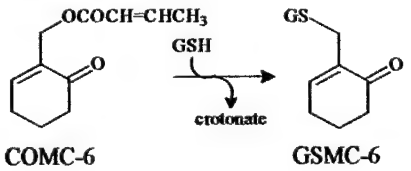
The hypothesis that the antitumor activity of 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) is associated with the formation of its GSH conjugate (GSMC-6) inside tumor cells is inconsistent with recent experimental findings. The diethyl ester prodrug form of GSMC-6 displays little antitumor activity with B16 melanotic melanoma (IC_{50} > 460 μM) in culture. This result contrasts with the potency of COMC-6 (IC_{50} 0.041 μM) and its corresponding five- and seven-membered ring homologues COMC-5 (IC_{50} 0.14 μM) and COMC-7 (IC_{50} 0.029 μM). These and other observations suggest that antitumor activity of COMCs is linked to the formation of an electrophilic exocyclic enone intermediate formed during conjugation of the COMCs with GSH inside tumor cells.

The *Streptomyces* metabolite 2-crotonyloxymethyl - (4R,5R,6R)-4,5,6-trihydroxy-2-cyclohexenone (COTC) and its synthetic analog 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) are potent antitumor agents against both murine and human tumors in culture and in tumor-bearing mice.[1-3] As such, these compounds have attracted considerable interest as synthetic targets.[4-8] Early investigators proposed that antitumor activity might arise from competitive inhibition of the methylglyoxal detoxifying enzyme glyoxalase I (GlxI) by the covalent adducts arising from the S_N2 displacement of crotonate by intracellular glutathione (GSH), Scheme 1.[1,3]

Scheme 1



GlxI plays an important role in detoxifying intracellular methylglyoxal, which is formed during normal carbohydrate metabolism.[9,10] Indeed, tight-binding enediol analogue inhibitors of GlxI retard the growth of both murine and human tumors in culture and in tumor-bearing mice by causing the accumulation of intracellular methylglyoxal.[11,12] However, the inhibitors showing antitumor activity have K_i values in the nanomolar concentration range with human erythrocyte GlxI. In contrast, the GSH adducts of COTC and COMC-6 have recently been shown to be relatively poor competitive inhibitors of human erythrocyte GlxI, with K_i values in the 100-200 μM range.[13,14] Therefore, antitumor activity is unlikely to arise from inhibition of GlxI. However, this finding does not exclude the possibility that the GSH adducts are toxic to tumor cells by some other mechanism.

In order to test this possibility, we first prepared the [*glycyl,glutamyl*] diethyl ester of the COMC-6 adduct of GSH (GSMC-6$(Et)_2$).[15] The relative antitumor activity of this compound was then compared with that of COMC-6. GSMC-6$(Et)_2$ should indirectly deliver GSMC-6 into cells by a process involving diffusion across the cell membrane followed by esterase-catalyzed deethylation to give COMC-6 This prodrug strategy has previously been used to deliver enediol analogue inhibitors of Glx-I into tumor cells.[11]

Accumulation studies confirmed this prediction. B16 melanotic melanoma in tissue culture was incubated with 50 μM GSMC-6$(Et)_2$ or COMC-6 for 30 sec, 10, 30, 60, or 120 min. As a function of time, cell pellets were lysed with 70% aqueous ethanol and fractionated by RPHPLC (Water's C_{18}, μBondapak column, 7.8 i.d. x 300 mm). Good base-line separation of metabolites was achieved using 15% aqueous methanol as a running solvent. Incubation with COMC-6 showed significant accumulation of GSMC-6 at 30 sec (0.041 nmol/ 10^7 cells) and 10 min (0.074 nmol/ 10^7 cells). No GSMC-6 was detected at later times. Incubation with GSMC-6$(Et)_2$ showed significant GSMC-6 at 30 sec (0.037 nmol/10^7 cells, but not at later times.

While incubation of B16 cells with either of these species results in significant intracellular accumulation of GSMC-6, only COMC-6 shows dramatic antitumor activity with an IC_{50} value 10^4-fold that of GSMC-6$(Et)_2$, Fig 1.

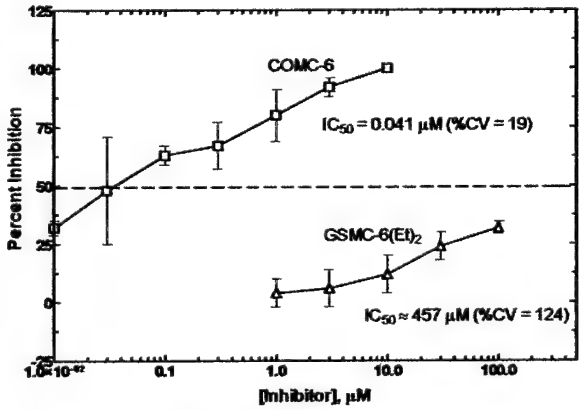


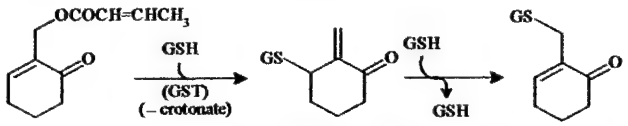
Figure 1. Growth inhibition of B16 cells in the presence of COMC-6 and GSMC-6$(Et)_2$. Methods: B16 (2 x 10^4 cells) were plated in 24 well plates containing RPMI 1640/10% bovine calf serum, 10 μg/ml gentamicin and incubated at 37°C under an atmosphere of 5% CO_2 and 95% humidified air. Drug was added at the indicated concentrations. After 72 h, cells were trypsinized, concentrated and counted by trypan blue exclusion using a hemocytometer. IC_{50} values are the mean ± standard deviation of triplicate determinations carried out in 3 separate assays on different days. IC_{50} values were calculated using the Hill equation and the program Adapt[16].

Therefore, the antitumor activity of COMC-6 cannot simply be due to the adduct GSMC-6, but must arise either directly from unconjugated COMC-6 or from an intermediate formed during the conjugation reaction between GSH and COMC-6.

With regard to the latter possibility, kinetic studies and intermediate trapping experiments showed that conjugation

results from a multi-step process involving Michael addition of GSH to COMC-6 to give a highly reactive exocyclic enone, which subsequently reacts with GSH in bulk solvent to give GSMC-6, Scheme 2.[14]

Scheme 2

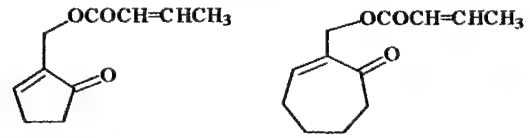


COMC-6 exocyclic enone GSMC-6

This was first revealed by the observation that human placental GST efficiently catalyzes the initial Michael addition reaction, giving rise to biphasic kinetics wherein the second step is rate determining. In the absence of enzyme, the rate constant for reaction of GSH with the exocyclic enone is about 12-fold larger than that for reaction of GSH with COMC-6. Therefore, the antitumor activity of COMC-6 could reasonably result from reaction of the exocyclic enone with proteins and/or nucleic acids critical to cell function. Indeed, this hypothesis is supported by mass spectral studies indicating that COMC-6 alkylates model oligonucleotides in the presence of GSH via a mechanism in which the exocyclic enone is probably the alkylating species.[17]

The respective 5- and 7-membered ring homologues of COMC-6 (Scheme 3) have also been shown to undergo conjugate additions with GSH that involve intermediate exocyclic enones.[18]

Scheme 3



COMC-5 COMC-7

Therefore, these species should also exhibit antitumor activity. Indeed, all three COMCs are toxic to B16 murine melanoma *in vitro,* with IC_{50} values indicating an increase in potency with increasing ring size, Fig 2. The similar shapes and parallel shift of the cytotoxicity curves suggest a similar mechanism of cytotoxicity for each compound. The IC_{50} values indicate an increase in potency with increasing ring size.

The cytotoxicity of COMC-6 was also examined in HT29(wt) human colon adenocarcinoma versus HT29 (MDR) cells that over express p-glycoprotein. This experiment was prompted by a previous observation that COTC displays enhanced toxicity against certain types of drug-resistant neoplastic cells versus wild type cells.[19] In the present study, the IC_{50} value for HT29 (wt) cells was 0.80 μM while that for HT29 (MDR) was 1.8 μM, a change of only 2-fold, Fig. 3.

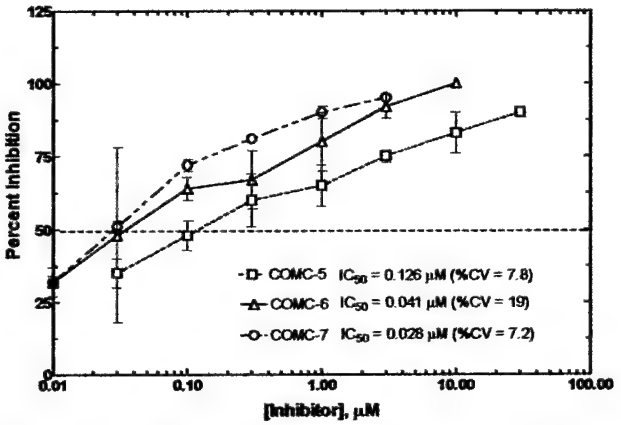


Figure 2. Growth inhibition of B16 cells in the presence of COMC-5, COMC-6 and COMC-7. Conditions/methods as in Fig.1.

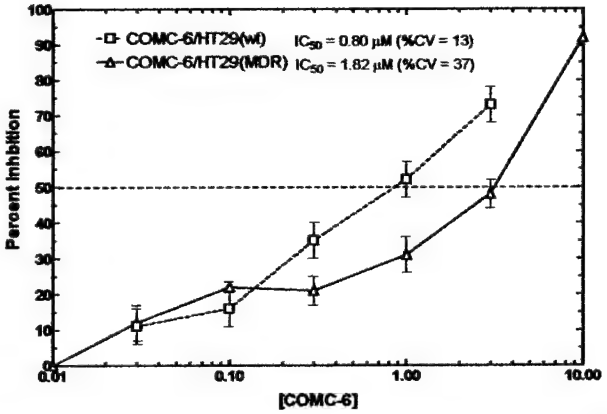


Figure. 3. Growth inhibition of HT29 (wt) versus HT29 (MDR) cells in the presence of COMC-6. Conditions/methods as in Fig. 1.

By comparison, a 17-fold difference in cytotoxicity of vincristine against these cell lines was noted in this laboratory: HT29 (wt), IC_{50} = 0.001 μM (%CV = .01); HT29 (MDR), IC_{50} = 0.017 μM (%CV = 17).

The central outcome of this work is that the GSH conjugate of COMC-6 (GSMC-6) can not be responsible for the antitumor activity of COMC-6, contrary to previous suggestions. The accumulation studies show the transient appearance (<30 min) of GSMC-6 in B-16 cells incubated in the presence of either COMC-6 or GSMC-6$(Et)_2$. The short life time of GSMC-6 inside B-16 cells is rather surprising, given that GSMC-6 is stable in aqueous solution for hours. One reasonable possibility is that GSMC-6 is reacting with nucleophilic species in the cytosol of the cell that are not critical for cell survival, as GSMC-6 is itself a Michael acceptor. The much greater toxicity of COMC-6 versus GSMC-6$(Et)_2$ is consistent with our previously proposed hypothesis that the exocyclic enone, formed as an intermediate during the conjugation reaction between GSH and the COMC-6, accounts for the difference in tumoricidal activities.[20] The cytotoxicity of COMC-5 and COMC-7 can be explained on the same basis.

The mechanism of cytotoxicity is not clear, but probably involves alkylation of protein(s) and/or nucleic acid(s) critical to cell function. The cytotoxic species also appears to be a poor substrate for the MDR-associated p-glycoprotein, given the similar IC_{50} values of COMC-6 with HT29 (wt) versus HT29 (MDR). This finding might best be rationalized by the fact that the glutathionylated exocyclic enone is multiply charged, and that p-glycoprotein most readily transports uncharged, hydrophobic antitumor agents.[21] This hypothesis might help to explain a previous observation that adriamycin-, aclarubicin-, and bleomycin-resistant sublines of murine lymphoblastoma L5178Y cells are no less sensitive to COTC than the parental cell line.[19] The possibility that GST plays a role in the antitumor activities of COMC derivatives is currently under investigation in our laboratories.

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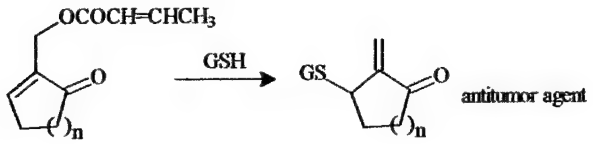
references

(1) Takeuchi, T.; Chimura, H.; Hamada, M.; Umezawa, H.; Yoshioka, O.; Oguchi, N.; Takahashi, Y.; Matsuda, A. A Glyoxalase I Inhibitor of a New Structural Type Produced by *Streptomyces*. *J. Antibiot.* **1975**, *28*, 737-742.
(2) Chimura, H.; Nakamura, H.; Takita, T.; Takeuchi, T.; Umezawa, H.; Kato, K.; Saito, S.; Tomisawa, T.; Iitaka, Y. The Structure of a Glyoxalase I Inhibitor and its Chemical Reactivity with SH-Compounds. *J. Antibiot.* **1975**, *28*, 743-748.
(3) Aghil, O.; Bibby, M.C.; Carrington, S.J.; Double, J.; Douglas, K.T.; Phillips, R.M.; Shing, T.K.M. Synthesis and Cytotoxicity of Shikimate Analogues. Structure:Activity Studies Based on 1-Crotonyloxymethyl-3R,4R,5R-trihydroxycyclohex-2-enone. *Anti-Cancer Drug Design* **1992**, *7*, 67-82.
(4) Mirza, S.; Molleyres, L.-P.; Vasella, A. Synthesis of a Glyoxalase I Inhibitor from *Streptomyces griseosporeus* Niida *et* Ogasawara. *Helv. Chim. Acta* **1985**, *68*, 988-996.
(5) Takayama, H.; Hayashi, K.; Koizumi, T. Enantioselective Total Synthesis of Glyoxalase I Inhibitor Using Asymmetric Diels-Alder Reaction of a New Chiral Dienophile, (S)$_S$-3-(3-Trifluoromethylpyrid-2-ylsulfinyl)-acrylate. *Tetrahedron Lett.* **1986**, *27*, 5509-5512.
(6) Shing, T.K.M.; Tang, Y. (-) Quinic Acid in Organic Synthesis. 1. A Facile Synthesis of 2-Crotonyloxymethyl-(4R,5R,6R)-4,5,6-trihydroxy-cyclohex-2-enone. *Tetrahedron* **1990**, *46*, 6575-6584.
(7) Tatsuta, K.; Yauda, S.; Atraki, N.; Takashashi, M.; Kamiya, Y. Total Synthesis of a Glyoxalase I Inhibitor and Its Precursor, (–)-KD16-U1. *Tetrahedron Lett.* **1998**, *39*, 401-402.
(8) Huntley, C.F.M.; Wood, H.B.; Ganem, B. A New Synthesis of the Glyoxalase-I Inhibitor COTC. *Tetrahedron Lett.* **2000**, *41*, 2031-2034.
(9) Creighton, D.J.; Pourmotabbed, T. In *Molecular Structure and Energetics: Principles of Enzyme Activity*; Liebman, J.F., Greenberg, A. Eds.; VCH Publishers; New York, 1988; Vol. 9, pp 353-386.
(10) Richard, J.P. Kinetic Parameters for the Elimination Reaction Catalyzed by Triosephosphate Isomerase and an Estimation of the Reaction's Physiological Significance. *Biochemistry* **1991**, *30*, 4581-4585.
(11) Kavarana, M.J.; Kovaleva, E.G.; Creighton, D.J.; Wollman, M.B.; Eiseman, J.L. Mechanism-Based Competitive Inhibitors of Glyoxalase I: Intracellular Delivery, In Vitro Antitumor Activities, and Stabilities in Human Serum and Mouse Serum. *J. Med. Chem.* **1999**, *42*, 221-228.
(12) Sharkey, E.M.; O'Neill, H.B.; Kavarana, M.J.; Wang, H.; Creighton, D.J.; Sentz, D.L.; Eiseman, J.L. Pharmacokinetics and Anti-Tumor Properties in Tumor Bearing Mice of an Enediol Analog Inhibitor of Glyoxalase I. *Cancer Chemother. Pharmacol.* **2000**, *46*, 156-166.
(13) Huntley, C. F.; Hamilton, D. S.; Creighton, D. J.; Ganem, B. Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor. *Organic Lett.* **2000**, *2*, 3143-3144.
(14) Hamilton, D. S.; Ding, Z.; Ganem, B.; Creighton, D.J. Glutathionyl Transferase Catalyzed Addition of Glutathione to COMC: A New Hypothesis for Antitumor Activity. *Org. Lett.* 2002, *4*, 1209 - 1212.
(15) The diethyl ester was prepared by incubating GSMC-6 with 2 N ethanolic HCl for 2h at room temperature. The solvent was removed in vacuo and the residue fractionated by reverse phase HPLC (Waters C_{18} μBondapak column, 7.8 i.d. x 300 mm) using methanol:water (25:75, v/v) containing 0.25% acetic acid as a running solvent. The desired product has a retention time of ~ 29 min at a flow rate of 2 ml/min; yield 75%. GSMC-6 was prepared by reacting COMC-6 with GSH in phosphate buffer (pH 7), following previously published procedures.[13,14] The 300 MHz ^{1}H-NMR spectrum (D_2O/DSS) of GSMC(Et)$_2$ featured the expected ethyl and glutathionyl resonances and the down field resonance (δ 7.13) characteristic of H3 in β,γ-unsaturated 2-cyclohexenones. FAB/MS of GSMC(Et)$_2$ gave the expected molecular ion, $(M+H)^+$= 472.
(16) D'Argenio, D.Z.; Schumitzky, A. A Package Program for Simulation and Parameter Estimation in Pharmacokinetic Systems. *Comput. Methods Programs Biomed.* **1979**,*9*, 115-134.
(17) Zhang, Q.; Ding, Z.; Creighton, D.J.; Ganem, B.; Fabris, D. Alkylation of Nucleic Acids by the Antitumor Agent COMC. Org. Lett. **2002**, *4*, 1459-1462.
(18) These compounds were synthesized by an adaptation of the procedure for preparing COMC-6.[14] Hamilton, D. S.; Ding, Z.; Zhang, X.; Hubatsch, I.; Mannervik, B.; Houk, K.N.; Ganem, B.; Creighton, D. J., unpublished.
(19) Sugimoto, Y.; Suzuki, H.; Yamaki, H.; Nishimura, T.; Tanaka, N. Mechanism of Action of 2-Crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone, a SH-Inhibitory Antitumor Antibiotic, and its Effect on Drug-Resistant Neoplastic Cells. *J. Antibiot.* **1982**, *35*, 1222-1230.
(20) A direct test of this hypothesis would be to deliver the exocyclic enone (Scheme 2) into B16 cells as the conjugate of the diethyl ester of GSH and test whether this diethyl ester is indeed more toxic to tumor cells than GSMC-6(Et)$_2$. However this experiment could not be done, because of the instability of the exocyclic enone in buffered solution at pH 7.
(21) Zamora, J. M.; Pearce, H. L.; Beck, W.T. Physical-chemical Properties Shared by Compounds that Modulate Multidrug Resistance in Human Leukemia Cells. *Mol. Pharmacol.* **1988**, *33*, 454-462.

Table of Contents Graphic :



Mechanism of the Glutathione Transferase-Catalyzed Addition of Glutathione to the Antitumor 2-Crotonyloxymethyl-2-cycloalkenones[¥]

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TITLE RUNNING HEAD: Processing of cycloalkenones by glutathione transferase

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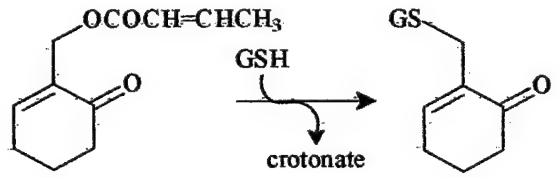
Abstract: The antitumor endocyclic enones 2-crotonyloxymethyl-2-cyclopentenone (COMC-5), 2-crotonyloxy-methyl-2-cyclohexenone (COMC-6) and 2-crotonyloxymethyl-2-cycloheptenone (COMC-7) have been synthesized and evaluated as substrates for several different isozymes of glutathione transferase (GST). Steady state kinetic measurements and intermediate trapping studies show that the hGSTP1-1 isoform catalyzes an S_N2' addition-elimination reaction to give highly reactive 3-glutathionyl 2-methylene cycloalkanones. These then dissociate from the active site and react with GSH in bulk solvent to give the final 2-glutathionylmethylcyclo-2-alkenones. Molecular docking studies using the X-ray structure of the human P1-1 isozyme suggest that Tyr 108 functions as a general acid catalyst by H-bonding with the carbonyl oxygen of bound endocyclic enone during the anti-addition of GSH. The steady-state kinetic constants for COMC-6 with hGST P1-1, A1-1, A4-4 and M2-2 are in the ranges: K_m = 0.08-0.34 mM, k_{cat} = 1.5 – 6.1 s^{-1}. No activity was detected with the rGST T2-2 isozyme. The kinetic constants for COMC-5 and COMC-7 with hGST P1-1 are similar in magnitude to those of COMC-6. These observations are consistent with the hypothesis that the antitumor activities of the COMC derivatives could be influenced by intracellular GST activity.

KEYWORDS: glutathione transferases, 2-crotonyloxymethyl-2-cycloalkenones, substrate specificity, reaction mechanism, antitumor activity.

The naturally occurring *Streptomyces* metabolite 2-crotonyloxymethyl-(4R,5R,6R)-456-trihydroxy-2-cyclohexenone (COTC) and its synthetic analog 2-crotonyloxymethyl-2-cyclohexenone (COMC-6) are known to be potent antitumor agents for both murine and human tumors in culture and in tumor-bearing mice.[1-3] As such, these compounds have attracted considerable interest as synthetic targets.[4-8] Early investigators proposed that antitumor activity might arise from competitive inhibition of the methylglyoxal detoxifying enzyme glyoxalase I by the covalent adducts arising from the S_N2 displacement of crotonate by intracellular glutathione (GSH), Scheme 1.[1] However this hypothesis was never explicitly tested.

Scheme 1

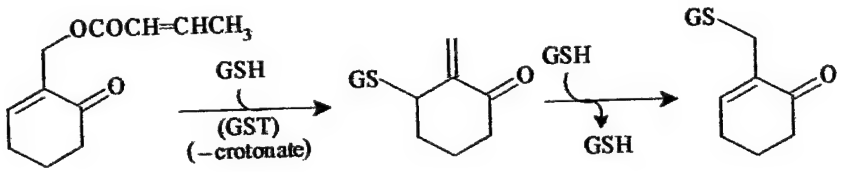


COMC-6 GSMC-6

The subsequent development of efficient synthetic routes to COMC-6 and its derivatives allowed a careful experimental examination of this hypothesis.[9,10] Surprisingly, the GSH adducts of COTC and COMC-6 were found to be poor competitive inhibitors of human erythrocyte glyoxalase I, and GSMC-6 did not exhibit significant toxicity toward melanotic melanoma in tissue culture when delivered into cells as the diethyl ester prodrug.[11] Therefore, contrary to the above hypothesis, antitumor activity is unlikely to arise from inhibition of glyoxalase I.

Recently, an alternative hypothesis for antitumor activity was advanced, on the basis of the discovery that human placental glutathione transferase (GST) catalyzes the conjugation reaction shown in Scheme 1.[10] Kinetic studies and intermediate trapping experiments showed that this was a multistep process first involving enzyme-catalyzed addition of GSH to COMC-6 to give a highly reactive exocyclic enone product, which subsequently reacts with GSH in bulk solvent to give GSMC-6, Scheme 2.

Scheme 2.



COMC-6 exocyclic enone GSMC-6

This addition-elimination mechanism is in sharp contrast to the previously proposed S_N2 displacement mechanism and has important implications with respect to the antitumor properties of COMC-6 and its derivatives. In principle, hGSTP1-1 might play a key role in the antitumor activity of these compounds by promoting the formation of the corresponding exocyclic enones, which could react with proteins and/or nucleic acids critical to cell function. Indeed, this hypothesis is supported by mass spectral studies indicating that COMC-6 alkylates model oligonucleotides in the presence of GSH via a mechanism in which the exocyclic enone is probably the alkylating species.[12]

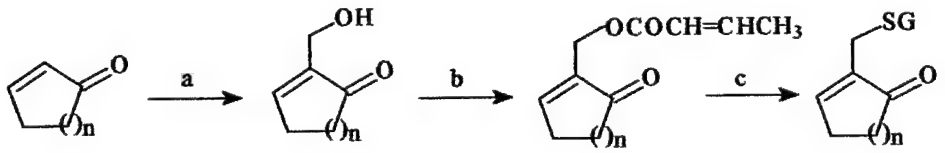
As part of a program aimed at using GST to generate antitumor agents inside cancer cells, we first describe the isozyme specificities and mechanistic features of the GST-catalyzed conjugation of GSH with COMC-6. The glutathione transferases (GST's) are a ubiquitous superfamily of enzymes that normally function to detoxify electrophilic xenobiotics by catalyzing their conjugation to glutathione (GSH).[13] Eight different classes of GST with a range of different substrate specificities have so far been identified: *alpha* (A), *pi* (P), *mu* (M), *kappa* (K), *sigma* (S), *theta* (T), *delta* (D) and *zeta* (Z). The *alpha*, *pi*, and *mu* GSTs are of particular interest from a cancer control perspective, as they are often over-expressed in the multi-drug resistance (MDR) tumor cells allowing them to rapidly detoxifying anticancer agents.[14] Therefore, a knowledge of the isozyme specificity for COMC derivatives could serve as a basis for selectively targeting tumor cells. Active site residues that might be involved in catalyzing the conjugation reaction between COMC-6 and GSH have been identified, on the basis of molecular docking studies. Also described is the synthesis and kinetic evaluation of the five- and seven-membered ring homologues of COMC-6 as substrates for *pi*GST.

MATERIALS AND METHODS

Enzymes. Human placental GSH transferase, which is primarily the P1-1 isozyme, was purchased from Sigma Chemical Co. and salts and free GSH were removed by ultrafiltration. The expression, isolation and purification of the other recombinant isozymes of GST used in this study have been described previously: hGST A1-1 [15]; hGSTA4-4 [16]; hGSTP1-1 [17]; hGSTM2-2 [18]; rGSTT2-2.[19]

Synthetic procedures. The synthesis of the 2-crotonyloxymethyl-2-cycloalkenones **1c – 3c** from the 2-cycloalkenones **1a – 3a** utilized the known Baylis-Hillman reaction of commercially available 2-cycloalkenones with formaldehyde to prepare the 2-hydroxymethyl-2-cycloalkenone **1b – 3b**,[20] which were then treated with crotonic anhydride to give **1c – 3c** (Scheme 2).[3]

Scheme 2. *Synthetic route to COMC derivatives (**1a-3a**) and their GSH adducts (**1c-3c**)*



1a, n = 1	**1b**, n = 1	**1c**, n = 1 (COMC-5)	**1d**, n = 1 (GSMC-5)
2a, n = 2	**2b**, n = 2	**2c**, n = 2 (COMC-6)	**2d**, n = 2 (GSMC-6)
3a, n = 3	**3b**, n = 3	**3c**, n = 3 (COMC-7)	**3d**, n = 3 (GSMC-7)

(a) DMAP, CH_2O, THF, rt; (b) crotonic anhydride, pyridine, DMAP, rt; (c) GSH, phosphate buffer (pH 7.5), 10 min.

The GSH conjugates **1d – 3d** (GSMC-5 – GSMC-7) were prepared from **1c – 3c** (COMC-5 – COMC-7) by incubation in the presence of GSH in buffered solution.[9] The NMR spectra of **1c – 3c** featured the expected glutathionyl resonances[21] and the characteristic downfield resonance (δ 7.12, triplet) characteristic of H-3 in β,γ-unsubstituted 2-cycloalkenones.

*2-Crotonyloxymethyl-2-cyclopentenone (COMC-5, **2a**).* 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 1.89 (dd, J= 1.5, 7.0 Hz, 3H), δ 2.46 (m, 2H), δ 2.64 (m, 2H), δ 4.83 (d, J = 1.5 Hz, 2H), δ 5.88 (dq, J= 1.5, 15.5 Hz, 1H), δ 7.02 (dq, J= 7.0, 15.5 Hz, 1H), δ 7.58 (m, 1H).

*2-Crotonyloxymethyl-2-cycloheptenone (COMC-7, **3a**).* 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 1.73-1.88 (m, 4H), δ 1.87 (dd, J= 1.8, 7.0 Hz, 3H), δ 2.46 (m, 2H), δ 2.64 (m, 2H), δ 4.81 (d, J = 1.1 Hz, 2H), δ 5.85 (dq, J= 1.8, 15.5 Hz, 1H), δ 6.79 (m, 1H), δ 6.98 (dq, J= 7.0, 15.5 Hz, 1H).

*2-Glutathionyl-2-cyclopentenone (**2c**).* 300 MHz ^{1}H NMR (D_2O, HOD ref): δ 2.14 (m, Glu-$C_\beta H_2$), δ 2.45-2.53 (m, Glu-$C_\gamma H_2$, ring CH_2), δ 2.66 (m, ring CH_2), δ 2.78 (q, J = 8.4, 13.9 Hz, Cys-$C_\beta H_b$), δ 2.96 (q, J = 4.8, 13.9 Hz, Cys-$C_\beta H_a$), δ 3.33 (s, CH_2), δ 3.80 (t, J = 6.4 Hz, Glu-C_α H), δ 3.95 (s, Gly-C_α H_2), δ 4.52 (q, J = 4.8, 8.4 Hz, Cys-C_α H), δ 6.92 (m, vinyl H).

*2-Glutathionyl-2-cycloheptenone (**3c**).* 300 MHz ^{1}H NMR (D_2O, HOD ref): δ 1.68-1.76 (m, ring 2xCH_2), δ 2.17 (m, Glu-$C_\beta H_2$), δ 2.43-2.56 (m, Glu-$C_\gamma H_2$, ring CH_2), δ 2.60 (m, ring CH_2), δ 2.77 (q, J = 8.8, 13.9 Hz, Cys-$C_\beta H_b$), δ 2.96 (q, J = 5.1, 13.9 Hz, Cys-$C_\beta H_a$), δ 3.35 (s, CH_2), δ 3.90 (t, J = 6.2 Hz, Glu-C_α H), δ 3.97 (s, Gly-C_α H_2), δ 4.52 (q, J = 4.8, 8.4 Hz, Cys-C_α H), δ 7.80 (t, J = 6.3 Hz, vinyl H).

Kinetic measurements. The kinetic constants associated with the GST-catalyzed conjugation of GSH with **1a-3a** were obtained from reciprocal plots of initial rates (ΔOD_{235}/min), versus [substrate], pH 6.5, [GSH] = 1 mM (25°C), under conditions where the enzyme-catalyzed step is rate limiting (< 0.08 units GST in the assay cuvette). Kinetic constants were obtained by curve-fitting the kinetic data with the software program Graphpad Prism.

Molecular docking. The structures of ethacrynic acid (EA), the GS-EA adduct, COMC-6 (**1a**), and the GS-**1a** adduct were minimized using MM3 or AMBER force field in MacroModel 7.1.[22] HF/6-31G* charges were assigned to atoms of all the structures for AutoDock calculations. The *X*-ray crystal structures of hGSTP1-1 in complex with EA or with GS-EA (PDB code: 2GSS and 3GSS, respectively) were used in docking simulations after removing all crystallographic water molecules and EA or GS-EA from the active sites. The Lamarckian genetic algorithm[23] was used in all the AutoDock calculations. One hundred twenty simulations were performed for each system. Each simulation was composed of a maximum of 500,000 energy evaluations and a maximum of 50,000 generations. Both rigid docking

. (deleting all rotatable torsion angles) and flexible docking (allowing all rotatable torsion angles) were applied.

RESULTS AND DISCUSSION

Substrate specificity for different classes of GST. Previously we showed that commercially available human placental GST, composed primarily of two *pi* enzyme variants with valine or isoleucine at position 104,[24] catalyzes the conversion of COMC-6 to GSMC-6, Scheme 1.[10] Under conditions where the enzyme-catalyzed step is completely rate limiting (< 0.08 units GST, with CDNB), $k_{cat} = 1.2 \pm 0.2\ s^{-1}$, $K_m = 0.052 \pm 0.01$ mM, GSH (1 mM), pH 6.5, 25°C. This is comparable to the activity of the enzyme with ethacrynic acid under the same assay conditions ($k_{cat} = 1.0 \pm 0.03\ s^{-1}$, $K_m = 0.034 \pm 0.007$ mM), suggesting that the two substrates are processed by the enzyme in the same way.

The kinetic properties of COMC-6 with recombinant enzymes of the *alpha*, *pi*, *mu* and *theta* classes were also evaluated, Table 1.

Table1. *Steady-state Kinetic Constants for COMC-6 with Different Classes of Glutathione Transferase (GST).*[a]

Isoform	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	[COMC-6] range (mM)
hGSTA1-1	1.5 ± 0.2 (0.04)[b]	0.13 ± 0.03	12 ± 2	0.025 – 0.280
hGSTA4-4	[c] (1.2)[b]	[c]	46 ± 6	0.013 – 0.280
hGSTP1-1(V105)	2.3 ± 0.2 (0.5)[b]	0.08 ± 0.01	30 ± 3	0.013 – 0.150
hGSTM2-2	6.1 ± 0.8 (0.09)[b]	0.34 ± 0.07	18 ± 1.3	0.013 – 0.280
rGSTT2-2	ND (0.18)[b]	ND	ND	0.1

[a] Conditions: GSH (1 mM), sodium phosphate (100 mM, pH 6.6), 30°C , $\Delta\varepsilon_{235} = -2400\ M^{-1}\ cm^{-1}$;

[b] k_{cat} values for ethacrynic acid (EA) estimated from published specific activities and subunit molecular weights.[2]

[c] saturation not achieved.

ND, no detectable activity; i.e., < 0.05 μmol min^{-1} mg^{-1} at 100 mM COMC-6.

All of these enzyme forms catalyze the conjugation of GSH to the exocyclic enone ethacrynic acid. The endocyclic enone COMC-6 also shows significant activity with the *alpha*, *pi*, *mu* enzyme classes with kinetic constants that are within a factor of five of one another. This probably reflects underlying similarities in the chemical mechanisms for the catalyzing conjugation of GSH with the enones. Crystal structure and site-directed mutagenesis studies show that hGSTP1-1, hGSTM2-2, and hGSTA1-1, have a conserved Tyr residue near the sulfur atom of bound GSH, which could stabilize the anion form of the glutathionyl sulfur (via H-bonding), thus, promoting nucleophilic addition to bound enones.[25,26] A different active site Tyr residue has been implicated to play an electrophilic role in catalysis by hGSTP1-1 (Y106) and hGSTM2-2 (Y115) with some substrates. Structural studies indicate that Y115 is well positioned to catalyze ring opening of bound phenanthrene 9,10 dioxide by H-bonding with the epoxide ring oxygen. In analogy, catalysis of the conjugation reaction between COMC-6 and GSH could involve H-bonding between the active site Tyr residues and the carbonyl oxygen of bound COMC-6.

In the case of hGSTA4-4, the crystal structure of the enzyme in complex with S-(2-iodobenzyl)-glutathione suggests that Tyr212 could function in an electrophilic capacity during catalysis.[25,26] The enzyme is known to efficiently catalyze the Michael addition of GSH to 2-nonenal, and Tyr212 is well positioned to function as a general acid catalyst by interacting with the aldehydic oxygen.[27] Moreover, mutation of Tyr212 to Phe, Ser or Ala decreases the specific activity of the enzyme by about 20-fold. The hGSTA4-4 isozyme also catalyzes the addition of GSH to ethacrynic acid, a process that is dependent upon Tyr212. Therefore, it seems reasonable that COMC-6 is processed by a similar reaction mechanism. With respect to the hGSTA1-1 isozyme, a serine residue in the active site is in a position

analogous to that of Tyr212 in the hGSTA4-4 isozyme. Presumably, this residue function as a general acid/base catalyst during the processing of COMC-6. It is not clear why COMC-6 is inactive with rGSTT1-1 while ethacrynic acid displays significant activity with the enzyme, Table 1.

Molecular docking studies. Docking of COMC-6 into the *X*-ray structure of hGSTP1-1 was undertaken in order to determine whether Y106 is a reasonable candidate to play an electrophilic role in catalysis during the conjugation of GSH to COMC-6. The hGSTP1-1 enzyme was selected for study because a high-resolution *X*-ray structure of the enzyme in complex with EA is available and the kinetic constants of the enzyme with COMC-6 and EA are similar in magnitude, Table 1.

In order to test the reliability of the AutoDock program, EA and the GSH adduct GS-EA were docked back into the binding pockets observed in the X-ray crystal structures (PDB codes: 2GSS and 3GSS, respectively). While rigid docking did not reproduce the binding mode of EA in 2GSS, the binding mode of the GS-EA conjugate in 3GSS is well reproduced, Fig. 1. This is probably bec ause the bulky GS-EA has only one favorable conformation which docks into the active site. EA was also docked into the GSH-3GSS complex. However, neither rigid nor flexible docking could reproduce the orientation of EA observed in the crystal structure of the GS-EA-3GSS complex. Water molecules in the binding pockets appear to play a very important role in the orientation of bound EA in the active site. The most likely explanation for the failure to replicate the orientation of EA observed in the *X*-ray structure is that the docking studies were performed with the active water molecules retained in the binding pockets. According to Parker and coworkers, there are seven and three water molecules inside the binding pockets of the EA-2GSS and GS-EA-3GSS complexes, respectively.[28] During docking, there is significant overlap between these water molecules and EA which could result in binding modes not observed in the *X*- ray structure. Nevertheless, AutoDock should reliably reproduce the binding modes of GSH conjugates in the active site.

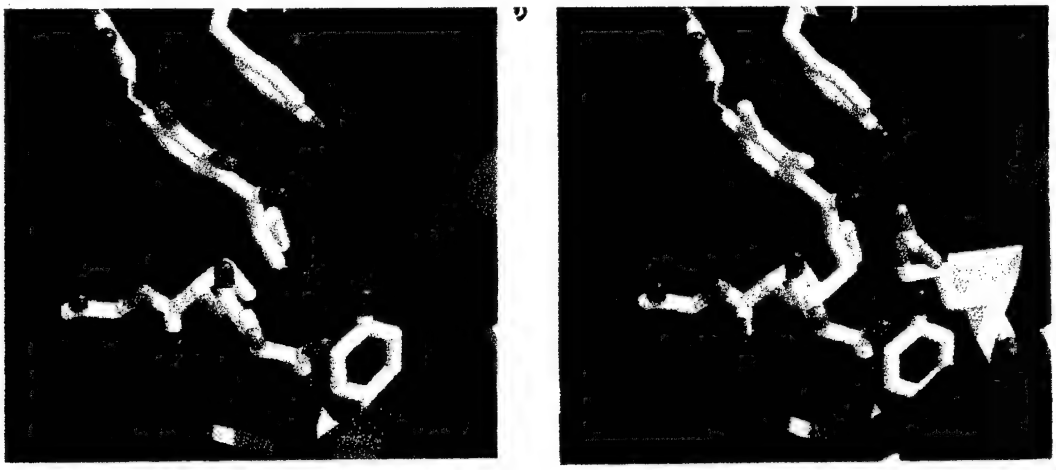
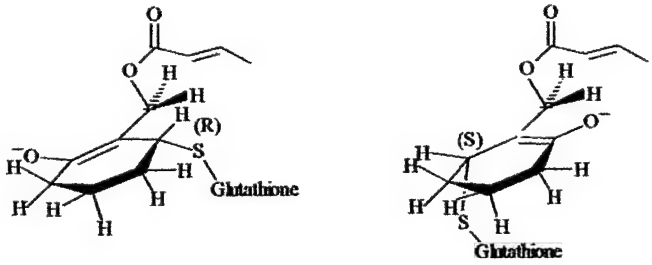


Figure 1. *Comparison of the structure of GS-EA derived from (a) the published X-ray structure of GS-EA in complex with 3GSS and (b) docking of GS-EA into the active site of 3GSS.*

Not surprisingly, docking of COMC-6 into the binding pocket of GS-3GSS did not provide reliable information about the probable mechanism of the proposed Michael addition reaction, as COMC-6 docks at a position distant from thiol anion of bound GS^-. However, useful information was obtained from docking studies with the two diastereomeric enolates, which result from addition of GSH to the 2si-3re face or to the 2re-3si face of COMC-6, Chart 1.

Chart 1



The diastereomers were first constructed in Macromodel 7.1 and then docked into 3GSS. The structure of the glutathionyl function was taken from the crystal structure and kept rigid during docking, as GSH has about the same conformation bound to different *pi* class transferases.[25] Therefore, only three rotatable bonds were allowed to change: one between the crotonate ether oxygen and the neighboring methylene C, one between the methylene C and the adjacent ring C, and the one between the glutathionyl S and the adjacent ring C. The results are shown in Fig. 2 and agree best with an anti-

addition-elimination mechanism involving the formation of the 3-(R) enolate, given that this structure is about 1 Kcal more stable than that of the bound 3-(S) diastereomer.

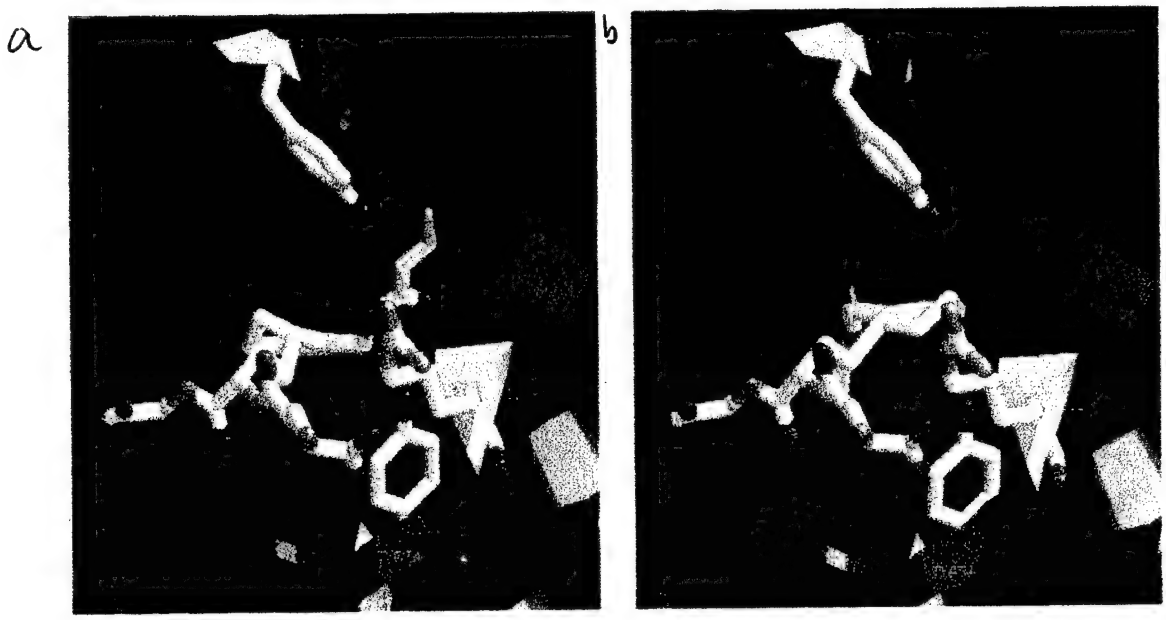


Figure. 2. *Lowest-energy binding modes of the (a) 3-R and (b) 3-S diastereomers of the enolates shown in Chart 2 bound to the active sites of 3GSS.*

Kinetic studies. Previously, we suggested that the known antitumor activity of COMC-6 could be due to a reactive exocyclic enone formed during the conjugation reaction with COMC-6, Scheme 1. Recently, we observed that not only COMC-6, but also the five and seven membered ring homologues COMC-5 and COMC-7 are toxic to B-16 melanotic melanoma *in vitro* with IC_{50} values of 0.14, 0.041 and 0.029 μM, respectively.[11] In order to test whether the anti-tumor activities of COMC-5 and COMC-7 could be explained on the basis of the formation of a reactive exocyclic enone, the kinetic properties of these compounds with hGSTP1-1 were determined.

The following observations support the formation of an exocyclic enone during the conjugation of COMC-6 with GSH: In the absence of hGST, the reaction of excess GSH with COMC-6 follows a simple first order decay, showing no evidence of an intermediate species, Fig 3A. However in the presence of hGSTP1-1, the reaction rate profile conforms to a double exponential decay, Fig.3B. The magnitude of the rate constant associated with the initial downward phase, after subtracting the rate

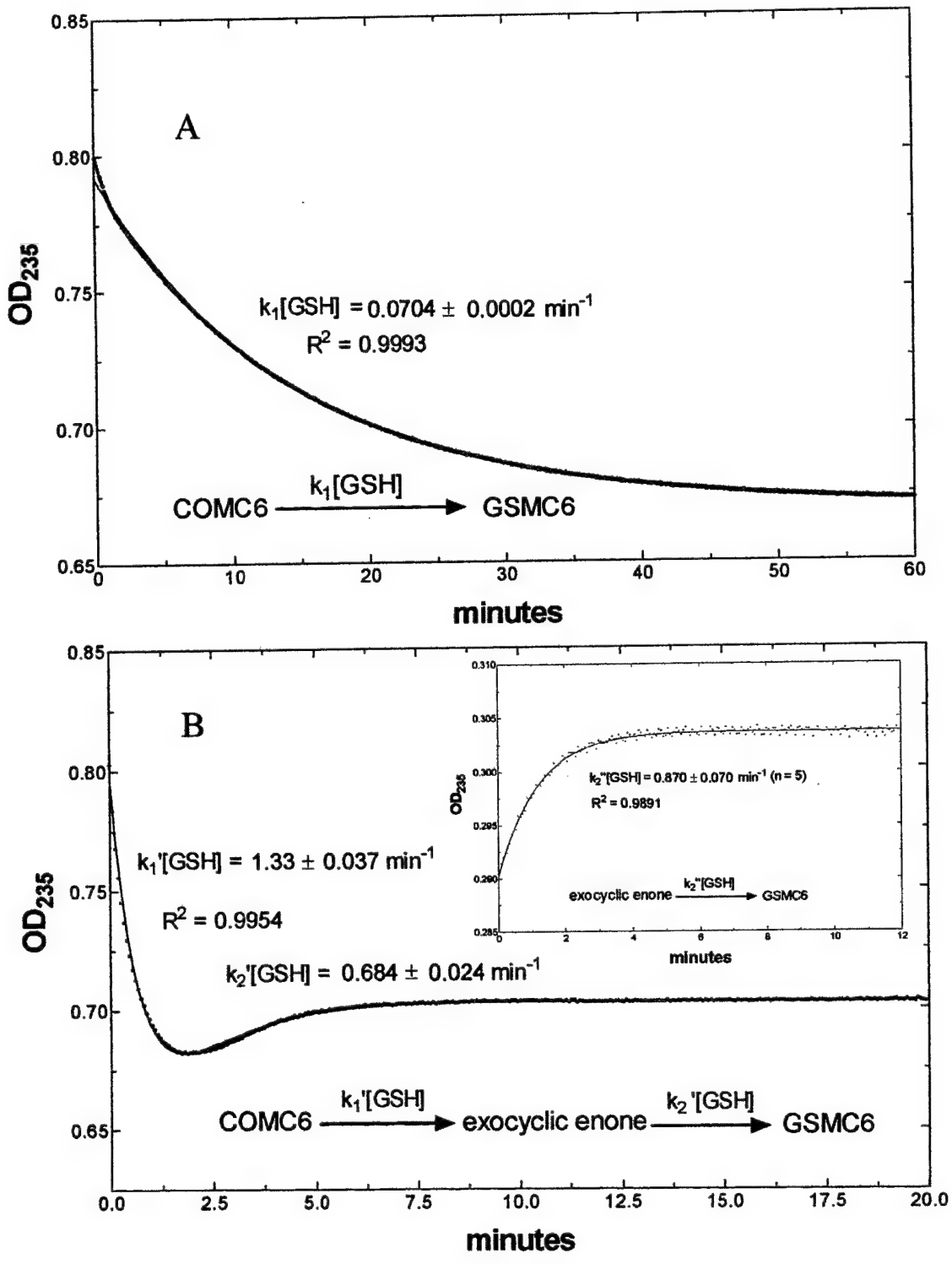


Figure 3. *Rate profile for the reaction of (A) COMC6 (0.05 mM) with GSH (1.05 mM),and (B) COMC6 (0.05 mM) with GSH (1.03 mM) in the presence of GST (2.4 CDNB units).* Rate constants were obtained as best-fit values to the equation $OD_{235} = OD_{235(t=inf.)} + a(\exp(-k_1't)) + b(\exp(k_2't))$. The insert in panel B shows the exponential rise in OD_{235} due to the reaction of

the isolated exocyclic enone with GSH (1.03 mM). *Conditions*: Potassium phosphate buffer (0.1 M, pH 6.5), EDTA (0.05 mM), 1% ethanol, 25°C.

constant for the nonenzymic addition of GSH with COMC-6, is linearly dependent on the concentration of hGSTP1-1 in the range 0 to 2.4 (CDNB) units of enzyme activity. The magnitude of the rate constant associated with the second, upward phase of the reaction is enzyme independent. These observations are consistent with enzyme-catalyzed formation of a reactive species that subsequently dissociates from the enzyme and reacts with GSH in bulk solvent to give GSMC-6.

This intermediate species was isolated by incubating COMC-6, GSH and hGSTP1-1 under the conditions described in Fig. 3 for about 1 min and resolving the reaction mixture by reverse-phase HPLC using a Water's μBondapak C_{18} column (7.8 mm i.d. x 30 cm, 10 μm; running solvent, ? % methanol in water). The retention time of the transient species (??? min) was close to that of GSMC-6 (??? min). The ^{1}H NMR spectrum showed the characteristic vinyl protons expected of an exocyclic enone, as well as the resonances expected of a glutathione moiety.[21] Incubation of the putative exocyclic enone with GSH gives a species that comigrates with authentic GSMC-6 by HPLC. Moreover, the spectrophotometrically determined pseudo first-order rate constant for reaction of the exocyclic enone with GSH is similar in magnitude to the second phase of the reaction of COMC-6 with GSH in the presence of hGSTP1-1, Fig 3B (inset). The absorptivity of the exocyclic enone ($\varepsilon = 4300$ cm^{-1} M^{-1}) is significantly less than that of GSMC-6 ($\varepsilon = 7500$ cm^{-1} M^{-1}), which accounts for the overall shape of the reaction rate profile in the presence of transferase.

The ring-expanded COMC-7 displays more complex kinetic behavior. In the absence of hGSTP1-1, the reaction of COMC-7 with excess GSH in aqueous buffer is best fit using a two-phase exponential decay, implying at least two sequential reactions, Fig 4A. Indeed, if the reaction is followed by using reverse-phase HPLC, wherein aliquots are removed from the reaction mixture as a function of time and resolved on a Water's μBondapak C_{18} column (7.8 mm i.d. x 30 cm, 10 μm; running solvent, ? % methanol in water), the peak corresponding to COMC-7 (???min) is rapidly transformed into a peak

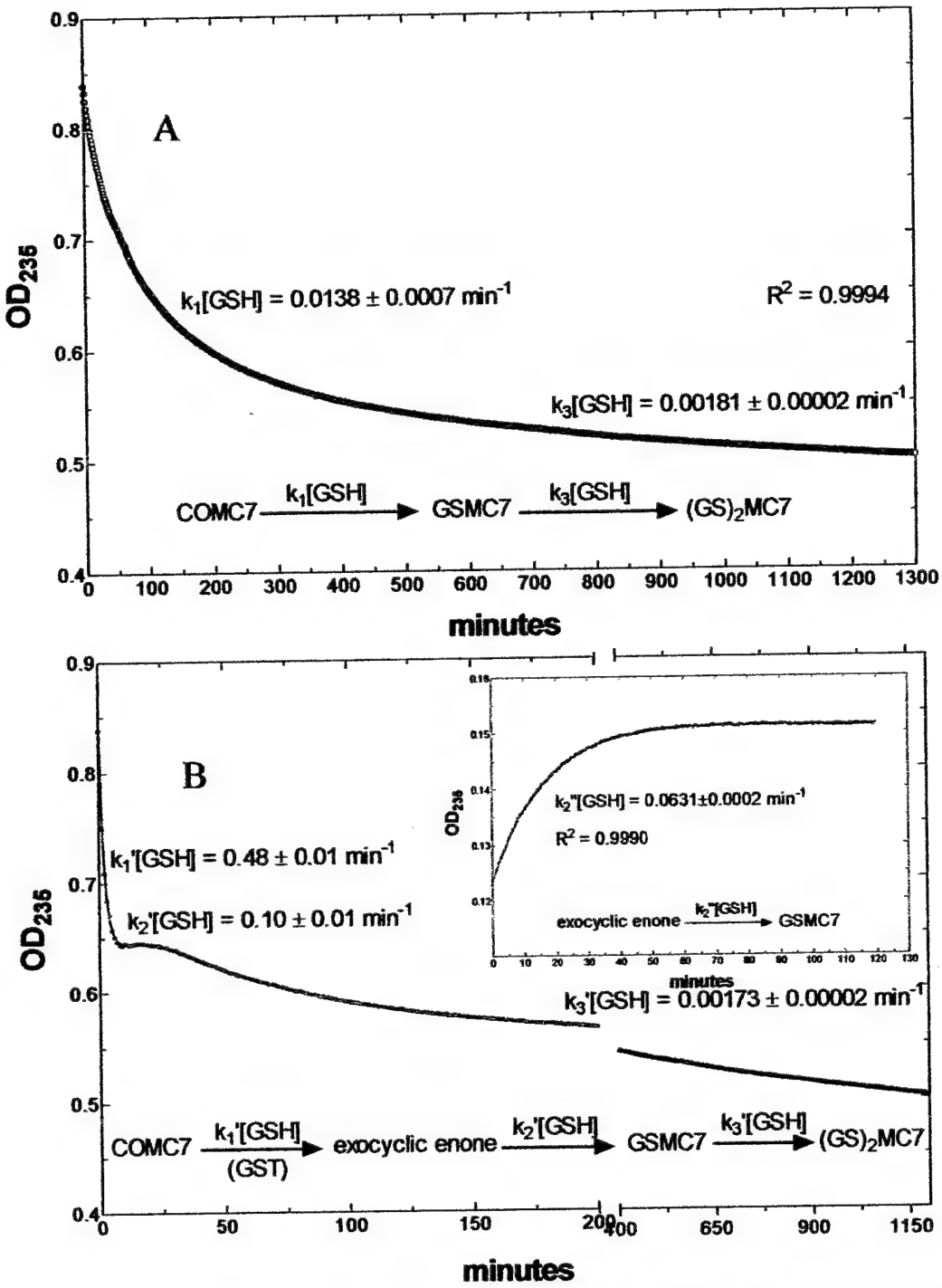
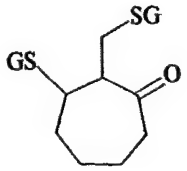


Figure 4. *Rate profiles for the reaction of (A) COMC-7 (0.05 mM) with GSH (1.05 mM), and (B) COMC-7 (0.05 mM) with GSH (1.05 mM) in the presence of GST (1 CDNB unit).* For (A) rate constants were obtained as best-fit values to the equation $OD_{235} = OD_{235(t=inf.)} + a(\exp(-k_1t)) + b(\exp(k_3t))$. For (B), rate constants were obtained as best-fit values to the equation $OD_{235} = OD_{235(t=inf.)} + a(\exp(-k_1't)) + b(\exp(k_2't)) + c(\exp(-k_3't))$. The inset in panel B shows the exponential rise in OD_{235} due to the reaction of the isolated exocyclic

enone with GSH (1.05 mM). *Conditions*: Potassium phosphate buffer (0.1 M, pH 6.5), EDTA (0.05 mM), 1% ethanol, 25°C.

which co migrates with GSMC-7 (21 min). After extended incubation (> 1300 min), the GSMC-7 peak is converted into four different peaks with retention times of ??? min, ???? min, ???? min, ???? min. The FAB mass spectra of the species associated with each peak gave identical molecular ions ($M + H^+$ = ???), consistent with the following structure:



$(GS)_2$MC-7

The H^1 NMR spectrum of the species corresponding to the major peak at ???? min. shows the characterisic resonances of the glutathionyl and cyclohexanone functions. Therefore, the final product species isolated by HPLC are likely the diastereomers of the diglutathione adduct $(GS)_2$MC-7, which differ from one another with respect to the configurations at C2 and C3.

In the presence of hGSTP1-1, the kinetic data could be fit to a triple exponential function, Fig. 4B. The magnitude of the observed rate constant (k_1'[GSH]) associated with the initial, rapid phase (after substracting the rate constant k_1[GSH] for the nonenzymic reaction) is linearly dependent on enzyme concentration from 0-1.0 CDNB units of hGSTP1-1. The rate constant k_2' appears to be associated with the conversion of the exocyclic enone to GSMC-7. Brief incubation of COMC-7, GSH and hGSTP1-1 gives rise to a transient species that was isolated by reverse-phase HPLC, under the conditions described above, with a retention time (26 min) close to that of GSMC-7 (21 min). ^{1}H NMR spectroscopy confirmed the identity of the exocyclic enone. Incubation of the exocyclic enone in the presence of GSH in buffered solution gives a species that comigrates with authentic GSMC-7 by HPLC. Moreover, the spectrophotometrically determined pseudo first-order rate constant for reaction of the exocyclic enone with GSH is similar in magnitude to the second phase of the reaction of COMC-6 with

GSH in the presence of hGSTP1-1, Fig 4B (inset). The slow decrease in absorbancy with a rate constant k_3'[GSH] correspond closely to k_3[GSH] for conversion of GSMC-7 to the diglutathione adduct $(GS)_2$MC-7

The ring-contracted compound COMC-5 displays kinetic properties similar to those of COMC-6. In the absence of hGSTP1-1, the reaction of excess GSH with COMC-6 follows a simple first order decay, Fig 5A. However in the presence of hGSTP1-1, the reaction rate profile conforms to a double exponential decay, Fig.4B. As with COMC-6, the magnitude of the rate constant associated with the initial downward phase, after subtracting the rate constant for the nonenzymic addition of GSH with COMC-6, is linearly dependent on the concentration of hGSTP1-1 in the range 0 to 1.0 (CDNB) units of enzyme activity. The magnitude of the rate constant associated with the second, upward phase of the reaction is enzyme independent. These observations are consistent with enzyme-catalyzed formation of an exocyclic enone that dissociates from the enzyme and subsequently reacts with GSH in bulk solvent to give GSMC-5. Brief incubation of COMC-5, GSH and hGSTP1-1 gives rise to a transient species that was isolated by reverse-phase HPLC under the condition described for isolating the transient species generated from COMC-6. As in the case of the COMC-6 and COMC-7 reactions, the retention time of this intermediate (17.6 min) derived from COMC-5 was slightly longer than that of GSMC-5 (15.8 min). Incubation of this species with GSH in buffered solution gives a species that comigrates with authentic GSMC-5 by HPLC. However, the rate constant associated with this reaction in the presence of about 1mM GSH (Fig. 5, inset) is nearly 30-fold larger than that associated with the slower, second phase of the reaction between COMC-5 and GSH in the presence of hGSTP1-1. HPLC analysis of the reaction at long times reveals that GSMC-5 is reacting further with GSH to give ???????

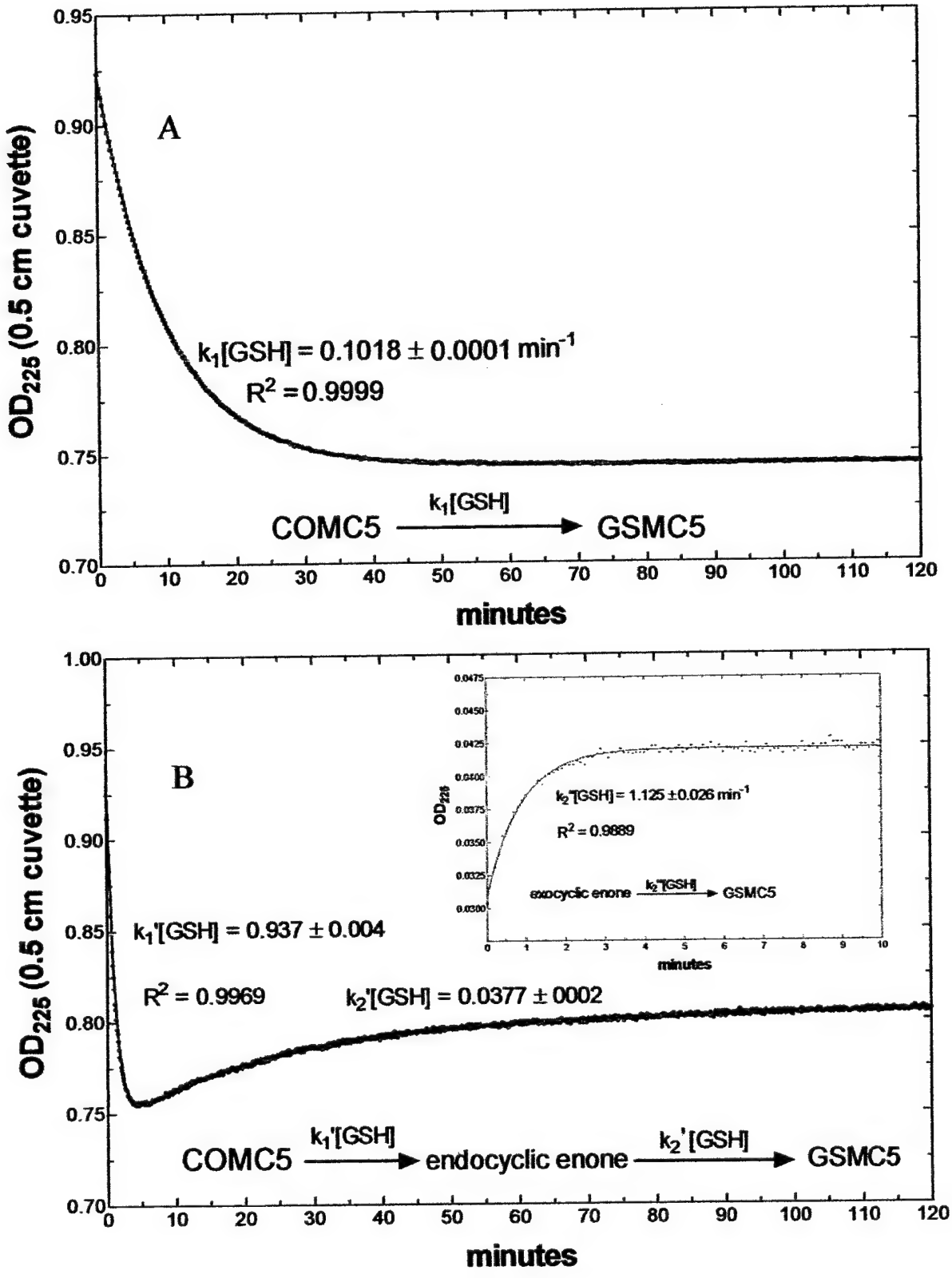


Figure 5. *Rate profiles for the reaction of (A) COMC-5 (0.05 mM) with GSH (1.05 mM), and (B) COMC-5 (0.05 mM) with GSH (1.05 mM) in the presence of GST (1 CDNB*

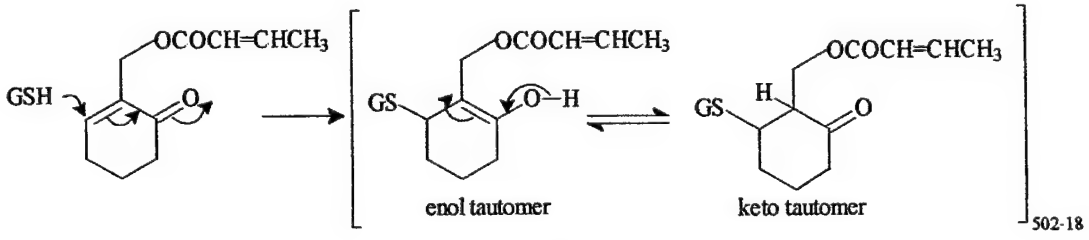
unit). Rate constants were obtained as best-fit values to the equation $OD_{235} = OD_{235(t=inf.)} + a(\exp(-k_1't)) + b(\exp(k_2't))$. The insert in panel B shows the exponential rise in OD_{225} due to the reaction of the isolated exocyclic enone with GSH (1.05 mM). *Conditions*: Potassium phosphate buffer (0.1 M, pH 6.5), EDTA (0.05 mM), 1% ethanol, 25°C.

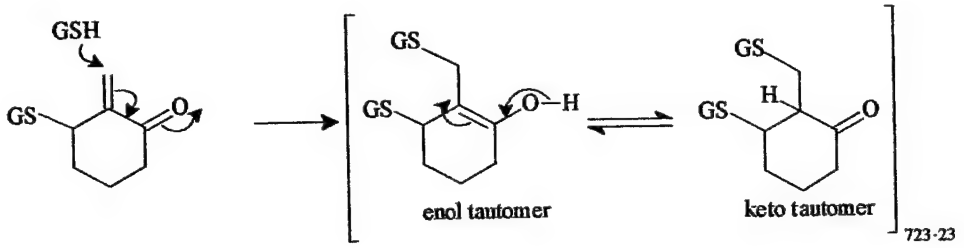
SUMMARY AND CONCLUSIONS

The work presented here supports the hypothesis that GST catalyzes a Michael addition of GSH to the endocyclic enones COMC-5, COMC-6 and COMC-7 to give reactive exocyclic enones, which subsequently reacts with GSH in bulk solvent to give the final GSH adduct. This conclusion is supported by the multiphasic kinetics observed in the presence of GST, and the actual isolation and kinetic characterization of the intermediate exocyclic enones.

Previously, the nonenzymic reaction of GSH with COMC-6 was thought to involve a simple S_N2 displacement of crotonate by GSH.[3] Indeed, the reaction of excess GSH with COMC-6 follows a simple first order decay with no evidence of an intermediate species, Fig.3. Conceivably, the nonenzymic and GST-catalyzed reactions might proceed through different mechanisms, wherein the former reaction involves an S_N2 displacement mechanism and the latter involves a Michael addition. However, this seems less likely, in view of recent mass spectral studies of the nonenzymic reaction of excess COMC-6 with GSH.[12] Using electrospray Fourier transform mass spectrometry, $M+H^+$ ions were detected for the enolate species (or the corresponding keto tautomer) expected from (a) Michael addition of GSH to COMC-6 ($M+H^+$ 502.18) and (b) addition of GSH to the putative exocyclic enone intermediate ($M+H^+$ 723.23), Scheme 3.

Scheme 3





An $M+H^+$ ion was also observed corresponding to that of the exocyclic enone, but this species is redundant with that of GSMC-6. *Therefore, the simplest explanation for all of these observations is that the first-order kinetics observed for the reaction of excess GSH with COMC-6 in the absence of GST is due to rate-limiting formation of the exocyclic enone.* In the presence of high concentrations of GST, the formation of the intermediate is no longer completely rate-limiting and complex multiphasic kinetics are observed, Figs 3-4.

The mechanism-of-action of the GST-catalyzed Michael addition GSH to COMC-6 most likely involves an anti-addition-elimination, in which the hydroxyl group of Tyr 108 plays an electrophilic role in catalysis by H-bonding with the carbonyl oxygen of bound COMC-6. This conclusion is supported by the molecular docking studies and stereochemistry experiments. The fact that COMC is a reasonably good substrate for the *alpha, mu* and *pi* classes of GST probably reflects a fundamentally similar catalytic mechanism among these enzyme forms for this substrate. Indeed, the placement of catalytic residues with in their active sites is similar, on the basis of the *X*-ray crystal structures of the different isoforms. The fact that the hGSTA4-4 isozyme has the largest value of k_{cat}/K_m with COMC-6 is consistent with the proposal that this isoform has evolved to detoxify enones resulting from lipid peroxidation.[27]

What are the long-range implications of this work? In the past, a common rationale for doing structure and mechanism studies on the GSTs has been to provide a basis for developing inhibitors of the GSTs in order to overcome multidrug resistance in tumor cells. An altenantive strategy is to use the transferases to manufacture toxic molecules inside tumor cells over-expressing the enzyme, in order to selectively inhibit tumor cells versus normal cells. The only published example of the latter strategy is

that of Lyttle and coworkers, in which they demonstrated that GSTP1-1 catalyzes the release of toxic nitrogen mustards from the corresponding GSH conjugates.[29,30] We now report a second example of this general strategy, in which the *alpha, pi and mu* GSTs catalyze the formation of reactive exocyclic enones from the COMC derivatives that could alkylate proteins and nucleic acids critical to cell function,[12] potentially accounting for the antitumor activities of the COMC derivatives.[18] That COMC-6 is a good substrate for the *alpha, pi and mu* GSTs is important as these are the forms that are most often over expressed in multidrug resistant tumor cells.[31,32]

REFERENCES

1. Takeuchi, T., Chimura, H., Hamada, M., Umezawa, H., Yoshka, H., Oguchi, N., Takahashi, Y. and Matsuda, A. A. (1975) *J. Antibiot., 28*, 737-742.

2. Chimura, H., Nakamura, H., Takita, T., Takeuchi, T., Umezawa, M., Kato, K., Saito, S. Tomisawa, T. and Iitaka, Y. (1975) *J. Antibiot., 28*, 743-748.

3. Aghil, O., Bibby, M.C., Carrington, S.J., Doubic, J., Douglas, K.T., Phillips, R.M. and Shing, T.K.M.(1992) *Anti-Cancer Drug Design, 7*, 67-82.

4. Mirza, S., Molleyres, L.-P., and Vasella, A. (1985) *Helv. Chim. Acta, 68*, 988-996.

5. Takayama, H., Hayashi, K., and Koizumi, T. (1986) *Tetrahedron Lett. 27*, 5509-5512.

6. Shing, T.K.M., and Tang, Y. (1990) *Tetrahedron, 46*, 6575-6584.

7. Tatsuta, K., Yauda, S., Atraki, N., Takashashi, M., and Kamiya, Y. (1998) *Tetrahedron Lett. 39*, 401-402.

8. Huntley, C.F.M., Wood, H.B., and Ganem, B. (2000) *Tetrahedron Lett., 41*, 2031-2034.

9. Huntley, C.F.M., Hamilton, D.S., Creighton, D. J., and Ganem, B. (2000) *Tetrahedron Lett.*, 2, 3143-3144.

10. Hamilton, D. S., Ding, Z., Ganem, B. and Creighton, D.J. (2002). *Org. Lett.*, ~~in press~~ 4, 1209-1212.

11. Joseph, E., Eiseman, J. L., Hamilton, D.S., Tak, H., Ganem, B. and Creighton, D.J. (2002) *Proceeding of the American Association for Cancer Research*, in press

12. Zhang, Q., Ding, Z., Creighton, D. J., Ganem, B. and Fabris, D. (2002) Org. Lett., in press

13. Mannervik, B., Awasthi, Y.C., Board, P.G., Hayes, J. D., Di, I. C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W.R., Pickett, C. B., Sato, K., Widersten, M., and Wolf, C.R. (1992) *Biochem. J. 282*, 305-306.

14. Hayes, J.D. and Pulford, D.J. (1995) *Crit. Rev. Biochem. Mol. Biol. 30,* 445-600.

15. Stenberg, G., Björnestedt, R., and Mannervik, B. (1992) *Protein Expression Purif. 3*, 80-84.

16. Hubatsch, I., Ridderstrom, M. and Mannervik, B. (1998) *Biochem. J. 330*, 175-179.

17. Kolm, R. H., Stenberg, G., Widersten, M. amd Mannervik, B. (1995) *Protein Expression Purif. 6*, 265-271.

18. Johansson, A.-S., Bolton-Grob, R., and Mannervik, B.(1999) *Protein Expr. Purif. 17* , 105-112.

19. Jemth, P., Stenberg, G., Chaga, G., and Mannervik, B. (1996) *Biochem. J. 316*, 131-136

20. Rezgui, F., and El Gaied, M. M. (1998) *Tetrahedron Lett. 39*, 5965-5966.

21. Rabenstein, D.L., and Keire, D.A. (1989). In *Coenzymes and Cofactors: Glutathione* (Dolphin, D., Poulson, R., Avramovic, O., eds) Johm Wiley, New York. Vol. 3, Part A, pp 67-101.

22. Mohamadi, F., Richard, N.G.J., Liskamp, W.C., Lipton, M., Canfield, C., Chang, G., Hendrickson, T. and Still, W. C. (1990) *J. Comp. Chem., 11*, 440-??

23. Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., and Olson, A.J. (1998) *J. Comp. Chem. 19*, 1639-1662.

24. Zimniak, P., Nanduri, B., Pikula, S., Bandorowicz-Pikula, J., Singhal, S., Srivastava, K. and Awasthi, S. (1994) *Eur. J. Biochem. 224*, 893-899.

25. Salinas, A. E., and Wong, M. (1999) *Current Medicinal Chemistry 6*, 279-309, and references therein.

26. Dirr, H., Reinemer, P. and Huber, R. (1994) *Eur. J. Biochem. 220*, 645-661, and references therein.

27. Bruns, C. M., Hubatsch, I., Ridderstrom, M., Mannervik, B. and Tainer, J.A. J. Mol. Biol. (1999) 288, 427-439.

28. Oakley, A.J., Rossjohn, J., Bello, M.L., Caccuri, A.M., and Parker, M.W.(1999) Biochemistry 38, 13480 - ???, and references therein.

29. Lyttle, M. H., Satyam, A., Hocker, M.D., Bauer, K.E., Caldwell, C.G., Hui, H.C., Morgan, A.S., Mergia, A. and Kauvar, L. M.(1994) *J. Med. Chem. 37*, 1501-1507.

30. Satyam, A., Hocker, M.D., Kane-Maguire, K.A., Morgan, A.S., Villar, H.O., and Lyttle, M. H. (1996) *J. Med. Chem. 39*, 1736-1747.

31. Montali, J.A., Wheatley, J.B., Schmidt, D.E., Jr. (1995) *Cell Pharmacol. 2*, 241-247.

32. Kauvar, L.M. (1993) In *Structure and Function of Glutathione Transferases*, Tew, K., Picket, C., Mantle, T., Mannervik, B., Hayes, J., Eds. CRC Press, Boca Raton , FL, pp 251-263.

APPENDIX II. Patent applied for

B. Ganem, D.J. Creighton, D. Zheng, and D. S. Hamilton, "Enone Cancer Therapeutics" Tech No. AS202, filed 3/15/02 by Cornell University and the University of Maryland, Baltimore County.

APPENDIX III. Current C.V. for Donald J. Creighton.

CURRICULUM VITAE

DONALD J. CREIGHTON

Personal Data

Date of Birth: January 25, 1946
Place of Birth: Stockton, California

Education

B.S., 1968, California State University, at Fresno, California, Chemistry
Ph.D., 1972, University of California, Los Angeles, California, Biochemistry

Positions Held

1990-present	University of Maryland, Baltimore County, Professor, Chemistry
1981-1990	University of Maryland, Baltimore County, Associate Professor, Chemistry
1975-1981	University of Maryland, Baltimore County, Assistant Professor, Chemistry
1972-1975	Institute for Cancer Research, Philadelphia, Pennsylvania, postdoctoral Fellow, Biochemistry

Professional Activities

Member, Florida Biomedical Research Study Section, 2001
Member, Tumor Biochemistry and Endocrinology Study Section, American Cancer Society, 1996-2001
Consultant, Bioorganic Chemistry and Natural Products Study Section, NIH, 1995, 1996, 1999
Vice-chair, Research Committee, American Heart Association (MD),1993-1994
Member, American Heart Association (MD) Study Section, 1991-1994
Member, American Chemical Society
Member, Sigma Xi

Honors

Outstanding Service Award, American Cancer Society, 2001
2001 External Lecturer in Bioorganic Chemistry, Uppsala University, Uppsala, Sweden
External thesis examiner, Department of Biochemistry, Uppsala University, Uppsala, Sweden, 1997, 2000
Presidential Teaching Professor, 1993-1996
Designated Subroto Chaterjee Fellow of the American Heart Association, 1990-1991

Visiting Professor Fudan University, Shanghai, 1988
NIH postdoctoral fellow, 1972-1975

Current Funding

D.J. Creighton, U.S. Army Medical Research and Material Command (Breast Cancer Research Program), "Inhibition of Tumor Cells that Over-Express π GST," 7/1/99-6/30/02, $295,893.

D.J. Creighton, National Cancer Institute, "Inhibition of the Antitumor Target Glyoxalase I,"2/16/01-1/31/04, $957,372.

Patents

B. Ganem, D.J. Creighton, D. Zheng, and D. S. Hamilton, "Enone Cancer Therapeutics" Tech No. AS202, filed 3/15/02.

D.J. Creighton, D.S. Hamilton and M.J. Kavarana, "Competitive Inhibitors of Glyoxalase I and Method of Generating Inhibitors Inside Tumor Cells," United States Patent No. 5,969,174; 10/19/99.

D.J. Creighton and D.S. Hamilton, "Glutathione N-hydroxycarbamoyl Thioesters and Method of Inhibiting Neoplastic Growth," United States Patent No. 5,616,563, 4/1/97.

Publications

50. Joseph, E., Eiseman, J. L., Hamilton, D.S., Wang, H., Tak, H., Ganem, B. and Creighton, D.J." Molecular Basis of the Antitumor Activities of Crotonyloxymethyl-2-cyclohexenones."J. Med Chem. in press (2002).

49. Q. Zhang, Z.Ding, D. J. Creighton, B. Ganem, and Fabris D."Alkylation of Nucleic Acids by the Antitumor Agent COMC." Organic Lett., 4, 1459-1462 (2002).

48. D. S. Hamilton, Z. Ding, B. Ganem and D. J. Creighton,"Glutathione S-Transferase-Catalyzed addition of Glutathione to COMC: A New Hypothesis for Antitumor Activity." Organic Lett., 4, 1209-1212. (2002).

47. H. Fan, M.J. Kavarana and D.J. Creighton, "Active-Site-Directed Inactivation of Glyoxalase I by S-(4-Bromo-2,3-dioxobutyl)glutathione," J. Med. Chem., in press (2001).

46. D.S. Hamilton and D.J. Creighton, "Using GSTP1-1 to Generate Enediol Analogue Inhibitors of Glyoxalase I," in Chemico-Biological Interactions, Elsevier, 133, 355-359 (2001).

45. D.J. Creighton and D.S. Hamilton, "Brief History of Glyoxalase I and What We Have Learned About Metal Ion-Dependent, Enzyme Catalyzed Isomerizations," Arch. Biochem. Biophys., 387, 1-10 (2001).

44. A. Kalsi, M.J. Kavarana, T. Lu, D.L. Whalen, D.S. Hamilton, and D.J. Creighton, "Properties of the Hydrophobic Binding Pocket in the Active Site of the Anti-Tumor Target Enzyme Glyoxalase I," J. Med. Chem., 43, 3981-3986 (2000).

43. C.F.M. Huntley, D.S. Hamilton, D.J. Creighton, and B. Ganem, "Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor," Organic Letters, 2, 3143-3144 (2000).

42. E.M. Sharkey, H.B. O'Neill, E.G. Kovaleva, M.J. Kavarana, H. Wang, D.J. Creighton, D.L. Sentz, and J.L. Eiseman, "Pharmacokinetics and Antitumor Properties in Tumor Bearing Mice of an Enediol Analog Inhibitor of Glyoxalase I," Cancer Chemotherapy and Pharmacology, 46, 156-166 (2000).

41. D.J. Creighton, D.S. Hamilton, M.J. Kavarana, E.M. Sharkey, and J.L. Eiseman, "Glyoxalase Enzyme System as a Potential Target for Anti-tumor Drug Development," Drugs of the Future, 25, 385-392 (2000).

40. F.A. Shamsi, E.M. Sharkey, D.J. Creighton, and R.H. Nagaraj, "Maillard Reactions in Lens Protein: Methylglyoxal-Mediated Modifications in the Rat Lens," Experimental Eye Research, 70, 369-380 (2000).

39. A.D. Cameron, M. Ridderström, B. Olin, M.J. Kavarana, D.J. Creighton, and B. Mannervik, "The Reaction Mechanism of Glyoxalase I Explored by an X-ray Crystallographic Analysis of the Human Enzyme in Complex with a Transition State Analogue," Biochemistry 38, 13480-13490 (1999).

38. D.S. Hamilton, M.J. Kavarana, E.M. Sharkey, D.J. Creighton, and J.L. Eiseman, "A New Method for Generating Inhibitors of Glyoxalase I Inside Tumor Cells Using S-(N-Aryl-N-hydroxycarbamoyl)ethylsulfoxide," J. Med. Chem., 42, 1823-1827 (1999).

37. M.J. Kavarana, E.G. Kovaleva, D.J. Creighton, and J.L. Eiseman, "Mechanism Based Competitive Inhibitors of Glyoxalase I: Membrane Transport Properties, in vitro Antitumor Activities, and Stabilities in Human Serum and Mouse Serum," J. Med. Chem., 42, 221-228 (1999).

36. A.P. Saint-Jean, K.R. Phillips, D.J. Creighton, and M.J. Stone, "Active Monomeric and Dimeric Forms of *Pseudomonas Putida* Glyoxalase I: Evidence for 3D Domain Swapping," Biochemistry 37, 10345 (1998).

35. M.J. Shih, J.W. Edinger, and D.J. Creighton, "Diffusion-Dependent Kinetic Properties of Glyoxalase I and Estimates of the Steady-State Concentrations of Glyoxalase Pathway Intermediates in Glycolyzing Erythrocytes," Eur. J. Biochem., 244, 857 (1997).

34. Y. Lan, T.L. Lu, P.S. Lovett, and D.J. Creighton, "Evidence for a (TIM-Like) 'Catalytic Loop' Near the Active Site of Glyoxalase I," J. Biol. Chem., 270, 12957 (1995).

33. T.L. Lu, D.J. Creighton, M. Antoine, C. Fenselau, and P.S. Lovett, "Gene Encoding

Glyoxalase I from Pseudomonas putida: Cloning, Overexpression, and Sequence Comparisons with Human Glyoxalase I," Gene 150, 93-96(1994).

32. N.S.R.K. Murthy, T. Bakeris, M.J. Kavarana, D.S. Hamilton, Y. Lan, and D.J. Creighton, "S-(N-Aryl N-hydroxycarbamoyl)glutathione Derivatives are Tight-Binding Inhibitors of Glyoxalase I and Slow Substrates for Glyoxalase II," J. Med. Chem., 37, 2161 (1994).

31. D.S. Hamilton, and D.J. Creighton, "Inhibition of Glyoxalase I by the Enediol Mimic S-(N-Hydroxy-N-methylcarbamoyl)glutathione: The Possible Basis of a Tumor-Selective Anticancer Strategy," J. Biol. Chem., 267, 24933 (1992).

30. D.S. Hamilton, and D.J. Creighton, "Caution: The Glycyl-methyl and Glycyl-ethyl Esters of Glutathione are Substrates for Glyoxalase I," Biochim. Biophys. Acta., 1159, 203 (1992).

29. J. Li, M.K. Guha, and D.J. Creighton, "Enzyme Chemistry of Dithiohemiacetals: Synthesis and Characterization of S-D-Dithio-mandeloylglutathione as an Alternate Substrate for Glyoxalase I," Biochem. Biophys. Res. Commun., 181, 657 (1991).

28. X. Xie, and D.J. Creighton, "Synthesis and Initial Characterization of γ-L-Glutamyl-L-Allo-Thiothreonylglycine as Steric Probes of the Active Site of Glyoxalase I," Biochem. Biophys. Res. Commun., 177, 252 (1991).

27. D.J. Creighton, "Stereochemistry of Enzyme Catalyzed Reactions at Carbon," in The Enzymes, Vol. 19, P.D. Boyer and D.S. Sigman (eds.), pp. 324-421 (1990).

26. T. Pourmotabbed, M.J. Shih, and D.J. Creighton, "Alternate Forms of Bovine Liver Formaldehyde Dehydrogenase and Their Kinetic and Molecular Properties," J. Biol. Chem., 264, 17384 (1989).

25. M. Guha, D.L. Vander Jagt, and D.J. Creighton, "Diffusion-Dependent Rates for the Hydrolysis Reaction Catalyzed by Glyoxalase II from Rat Erythrocytes," Biochemistry 27, 8818 (1988).

24. D.J. Creighton, and T. Pourmotabbed, "Glutathione-Dependent Aldehyde Oxidation Reactions" in Molecular Structure and Energetics: Principles of Enzyme Activity, Vol. 9, pp. 353-386, J.F. Liebman and A. Greenberg (Eds.), VCH Publishers (1988).

23. D.J. Creighton, M. Migliorini, T. Pourmotabbed, and M. Guha, "Optimization of Efficiency in the Glyoxalase Pathway," Biochemistry 27, 7376 (1988).

22. T. Pourmotabbed, and D.J. Creighton, "Substrate Specificity of Bovine Liver Formaldehyde Dehydrogenase," J. Biol. Chem. 261, 1424 (1986).

21. J.D. Johnson, D.J. Creighton, and M.R. Lambert, "Stereochemistry and Function of Oxaloacetate keto-enol Tautomerase." J. Biol. Chem. 261, 4535 (1986).

20. D.J. Creighton, and M. Migliorini, "Active Site Ionizations of Papain: An Evaluation of the Potentiometric Difference Titration Method." Eur, J. Biochem. 156, 189 (1986).

19. D.J. Creighton, J.D. Johnson, and M.R. Lambert, "Stereochemistry and Function of Oxaloacetate Keto-enol Tautomerase," Fed. Proc., 45, 1502 (1986).

18. T. Pourmotabbed, and D.J. Creighton, "Substrate Specificity of Bovine Liver Formaldehyde Dehydrogenase," Fed. Proc., 45, 1499 (1986).

17. M. Migliorini, and D.J. Creighton, "Interconversion Rates of the Diasteriotopic Thiohemiacetal Substrates of Glyoxalase I," Fed. Proc., 45, 324 (1986).

16. C.E.F. Griffis, L.H. Ong, L. Buettner, and D.J. Creighton, "Nonstereospecific Substrate Usage by Glyoxalase I," Biochemistry 22, 2945 (1983).

15. D.J. Creighton, A. Weiner, and L. Buettner, "Hydrophobic Binding is Not an Independent Stereochemical Determinant in the Yeast Glyoxalase I Reaction," Biophysical Chemistry, 11, 265 (1980).

14. D.J. Creighton, and D.J. Schamp, "Solvent Isotope Effects on Tautomerization Equilibria of Papain and Model Thiolamines, "FEBS Letters, 110, 313 (1980).

13. D.J. Creighton, M.R. Gessouroun, and J.M. Heapes, "Is the Thiolate-Imidazolium Ion Pair the catalytically Important Form of Papain?," FEBS Letters, 110, 319 (1980).

12. K.M. Welsh, D.J. Creighton, and J.P. Klinman, "Transition State Structure in the Yeast Alcohol Dehydrogenase Reaction: The Magnitude of Solvent and α-Secondary Hydrogen Isotopes Effects," Biochemistry, 19, 2005 (1980).

11. A. Wandinger, and D.J. Creighton, "Solvent Isotope Effects on the Rates of Alkylation of Thiolamine Models of Papain," FEBS Letters, 116, 116 (1980).

10. M. Frohlich, D.J. Creighton, and D.S. Sigman, "Limiting Rates of Ligand Association to Alcohol Dehydrogenase," Arch. Biochem. Biophys., 189, 471 (1978).

9. D.S. Sigman, J. Hajdu, and D.J. Creighton,"Nonenzymic Dihydronicotinamide Reductions as Probes for the Mechanism of NAD^+-Dependent Dehydrogenases," Bioorganic Chemistry, Vol. IV (ed. E.E. Van Tamelen)(1977).

8. D.J. Creighton, J. Hajdu, and D.S. Sigman, "Model Dehydrogenase Reactions. The Zinc Ion Catalyzed Reduction of Chelating Aldehydes by N-Propyl-1,4dihydronicotinamides and Borohydride," J. Amer. Chem. Soc., 98, 4619 (1976).

7. D.J. Creighton, and I.A. Rose, "Oxaloacetate Decarboxylase Activity in Muscle is Due to Pyruvate Kinase," J. Biol. Chem., 1, 69 (1976).

6. D.J. Creighton, and I.A. Rose, "Studies on the Mechanism and Stereochemical Properties of

the Oxaloactate Decarboxylase Activity of Pyruvate Kinase," J. Biol. Chem., 1, 61 (1976).

5. J.P. Klinman, K.R. Welsh, and D.J. Creighton, "Solvent Isotope Effects in the Yeast Alcohol Dehydrogenase Reaction," Paper #16, Second International Symposium on Alcohol and Aldehyde Metabolizing Systems, Philadelphia, PA (1976).

4. D.J. Creighton, "Studies on the Catalyzed Borohydride Reduction of Pyruvate by Oxaloacetate Decarboxylase," Federation Proceedings, 33, 885 (1974).

3. D.J. Creighton, J. Hajdu, G. Mooser, and D.S. Sigman, "Model Dehydrogenase Reactions. Reduction of N-Methylacridinium Ion by Reduced Nicotinamide Adenine Dinucleotide and Its Derivatives," J. Amer. Chem. Soc., 95, 6855 (1973).

2. D.S. Sigman, G.W. Wahl, and D.J. Creighton, "Models for Metalloenzymes. Zinc Ion Catalyzed Phosphorylation of 1,10-Phenanthroline-2-carbinol by Adenosine Triphosphate," Biochemistry 11, 2236 (1972).

1. D.J. Creighton, and D.S. Sigman, "A Model for Alcohol Dehydrogenase. The Zinc Ion Catalyzed Reduction of 1,10-Phenanthroline-2-carboxaldehyde by N-Propyl-1,4-dihydronicotinamide," J. Amer. Chem. Soc., 93, 6314 (1971).

APPENDIX IV: final payroll

INHIBITION OF TUMOR CELLS THAT OVER-EXPRESS n-GST

Payroll Summary

Paule-Esther Badou
Ina Belling
Diana Hamilton
Avinash Kalsi
Vatsala Sagar
Ellen Sharkey
Heekyunh Tak
Zhe-Bin Zheng
Guozhang Zhu